# Soil bacterial community structure and functional responses across a long-term mineral phosphorus (Pi) fertilisation gradient differ in grazed and cut grasslands

Randall, Kate; Creamer, Rachel; Brennan, Fiona; Clipson, Nicholas; Griffiths, BS; Storey, Sean; Doyle, Evelyn

*Published in:*
Applied Soil Ecology

*DOI:*
10.1016/j.apsoil.2019.02.002

Print publication: 01/06/2019

*Document Version*
Peer reviewed version

# Soil bacterial community structure and functional responses across a long-term mineral phosphorus fertilisation gradient differ in grazed and cut grasslands.

Kate Randall[b,a], Rachel Creamer[d,c], Fiona Brennan[c], Nicholas Clipson[a], Bryan Griffiths[e,c], Sean Storey[a], Evelyn Doyle[a]

[a]School of Biology and Environmental Science and Earth Institute University College Dublin, Dublin 4, Ireland

[b]School of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, CO4 3SQ, UK

[c]Teagasc Environmental Research Centre, Johnstown Castle, Wexford, Ireland

[d]Soil Biology Group, Wageningen University and Research, The Netherlands.

[e]SRUC, Crop and Soil Systems Research Group, West Mains Road, Edinburgh, EH9 3JG, UK

kate.randall@essex.ac.uk*

fiona.brennan@teagasc.ie

Nicholas.clipson@ucd.ie

rachel.creamer@wur.nl

evelyn.doyle@ucd.ie

Bryan.Griffiths@sruc.ac.uk

sean.storey@ucd.ie

**Abstract**

Grasslands form a significant proportion of land used across the globe and their future management is important. The objective of this study was to compare the long-term impact of inorganic phosphorus (Pi) fertilisation rates (P0, P15 and P30 $ha^{-1}$ $yr^{-1}$) under two grass management trials (grazed vs. cut and removed) on soil physicochemical properties, microbial biomass, phosphomonoesterase activity, bacterial community structure and abundance of a phosphorus (P) mineralising gene (*phoD*). Under grazing, microbial biomass and soil phosphorus concentrations (total and Pi) generally increased with Pi fertilisation rate, accompanied by significant differences in bacterial community structure between unfertilised (P0) and P30 soil. At the cut and removed site, although Pi was significantly greater in P30 soil, P concentrations (total and Pi) did not increase to the same extent as for grazing, with microbial biomass and bacterial community structures unresponsive to Pi fertilisation. Despite differences in soil P concentrations (total and Pi) and microbial biomass between sites, the abundance of bacterial *phoD* increased with increasing soil Pi across both, while phosphomonoesterase activity decreased. Amplicon sequencing revealed significant differences in relative abundances of bacterial genera at the grazed site only. Despite this, Acidobacteria were the dominant bacterial phylum across both grasslands. The relative abundance of bacterial genera *Gp6* and *Gp16* increased significantly with Pi fertilisation under grazing, while *Bradyrhizobium,* as well as unclassified genus-type groups belonging to *Actinobacteria* and *Acidimicrobiales* significantly decreased with Pi fertilisation, suggesting potential roles in P mobilisation when soil Pi concentrations are low. This study highlights the importance of long-term Pi fertilisation rates and aboveground vegetation removal in shaping soil bacterial community structure and microbial biomass, which in turn may impact soil fertility and plant productivity within agricultural soils.

**Keywords:** *agricultural management; bacteria; grassland; phosphorus; soil*

*DW – dry weight, Pi – inorganic phosphorus, Soil WHC – soil water holding capacity*

## 1. Introduction

Grasslands are the second largest terrestrial biome on the planet, comprising 37% of available soil (excluding Antarctica and Greenland) (FAO, 2010; O'Mara, 2012). They provide key services such as the production of food crops and meat. They contribute to the provision of clean water and flood prevention and are essential for carbon sequestration (FAO, 2015). With the current world population of 7.6 billion projected to reach 9.8 billion by 2050 (UN, 2017), there is considerable focus on improving productivity from grasslands to ensure food security. The management of grasslands has steadily intensified across Europe (Isselstein et al., 2005) and typically involves combinations of increased fertilisation, sward cutting (Lemanski and Scheu, 2015) and grazing (Shange et al., 2012; de Vries et al., 2015).

The efficient and sustainable fertilisation of agricultural soils is a significant challenge, and until alternative methods match productivity levels, additions of mineral fertilisers to soil will remain necessary (Tilman et al., 2002). Mineral/inorganic phosphorus (Pi) fertilisation is a particular concern for agriculture, as the main resource in Pi fertiliser production is finite (Richardson and Simpson, 2011; Simpson et al., 2011). As demand increases and stocks deplete, considerable interest has focussed on improving phosphorus (P) use efficiency within agriculture (Gorazda et al., 2013; Sharma et al., 2013; Dotaniya and Meena, 2014).

Water soluble inorganic phosphorus (Pi) is the primary form in which plants and microorganisms take up P. Organic P is however the dominant form in temperate grassland soils (Liebisch et al., 2014; Stutter, 2015), ranging between 20-80% of the total soil P (Sharma et al., 2013). The high reactivity of phosphate ions in soil means concentrations of Pi available for uptake are generally low, at less than 1% of the total P (Sims and Sharpley,

93 2005; Sharma et al., 2013), often resulting in P as the growth limiting nutrient for plants
94 (Sharma et al., 2013). To compensate for this, over-fertilisation was common practice. A
95 need to reduce pollution however led to European legislation (COM 91/676/EEC, 1991; COM
96 2000⁄60⁄EC, 2000) that now regulates fertilisation rates across Europe. This has boosted
97 efforts to find alternative methods for increasing concentrations of available Pi in soil.

98 Microorganisms are key drivers of P cycling within the terrestrial environment. The
99 mechanisms used can be broadly classified as inorganic P solubilisation, or organic P
100 mineralisation. Microbial inorganic P solubilisation occurs primarily through release of
101 organic anions which causes precipitated P-metal complexes to solubilise, releasing Pi
102 (Osorio and Habte, 2013; Sharma et al., 2013). Bacteria capable of this mode of solubilisation
103 include members of the *Pseudomonadaceae Rhizobiaceae*, *Caulobacteraceau*,
104 *Comamonadaceae*, *Burkholderiaceae*, *Enterobacteriaceae* within the Proteobacteria
105 (Mander et al., 2012), and representatives of the phylum Actinobacteria (Mander et al.,
106 2012; Wakelin et al., 2012). Organic P mineralisation is the breakdown of organic P
107 compounds to eventually release of Pi. Within soil this is catalysed by a broad group of
108 enzymes called phosphatases, with phosphomonoesterases (EC 3.13.2) particularly
109 important as they hydrolyse monoester bonds to release Pi. Microbial phosphate
110 mineralisation has been observed by organisms belonging to $\alpha$- and $\gamma$- *Proteobacteria*, the
111 *Actinobacteria* (Tan et al., 2013), *Actinomycetales*, *Bacilliales*, *Gloeobacterales*,
112 *Planctomycetales* and *Rhizobiales* (Ragot et al., 2016). In addition, some microorganisms
113 improve plant P uptake indirectly by modifying root systems and forming symbioses with
114 plants (Richardson and Simpson, 2011).

115 Understanding how soil microorganisms respond to Pi fertilisation within agricultural
116 systems could develop fertiliser regimes less reliant upon Pi fertiliser inputs (Owen et al.,

2015). However, the complexity of P cycling pathways means studies focussing on microbial P cycling have lagged behind those of carbon (C) and nitrogen (N). Molecular biomarkers to target microbial groups associated with P cycling typically focus on organic P mineralisation and phosphatases (Kageyama et al., 2011; Tan et al., 2013; Fraser et al., 2015; Acuña et al., 2016; Ragot et al., 2016), with many studies targeting bacterial alkaline phosphatases (Fraser et al., 2015; Chabbra et al., 2013; Tan et al., 2013). Three gene families encode for bacterial alkaline phosphatases (*phoD*, *phoX* and *phoA*). Although all three families have been identified in environmental samples, *phoD* has been found to dominate metagenomic datasets from soil environments (Tan et al., 2013). To-date most research on microbial P cycling has focussed on aquatic environments, with *phoD* being the most common biomarker. Although there are a number of studies profiling soil microbial responses to a variety of P fertilisation treatments across a range of management scenarios (Azziz et al., 2012; Osorio and Habte, 2013; Sharma et al., 2013; Tan et al., 2013; Chen et al., 2014), most have used exaggerated application rates (Wakelin et al., 2012), compared inorganic and organic P amendments (Fraser et al., 2015), compared single Pi rates to unfertilised controls, or use combined N and P regimes (Ragot et al., 2016). Comparisons across Pi fertilisation gradients typical for rain fed temperate grasslands are lacking.

Land management practices other than fertilisation have been shown to impact nutrient levels in soil (Jangid et al., 2008; Keith et al., 2012; Neal et al., 2017). Although grazing and cutting both remove aboveground vegetation, grazing generates a significant pathway of nutrient re-introduction belowground that cutting and removing vegetation does not. Most N and P ingested by grazing mammals is returned to soil via excreta (Parson et al., 2013). For N and P, this can equate to 65 N kg $yr^{-1}$ and 13 P kg $yr^{-1}$ for a mature cow (EU, 2014), whereas 30% of ingested C is returned to soil this way (70% is lost via $CO_2$ and

$CH_4$ production) (Parson et al., 2013). The effects of cutting and removing aboveground vegetation is relatively understudied in grassland systems compared to other aboveground grassland management approaches (Poeplau et al., 2016). Lower rates of C sequestration have been observed in cut and removed systems compared to grazing (Fitter et al., 1997; Hungate et al., 1997; Nitschelm et al., 1997), but grazing effects on soil organic C within grassland soils can vary depending on context (McSherry and Ritchie, 2013; Abdalla et al., 2018). Considering the importance of grazing, cutting and fertilisation regimes in grassland management, an understanding of how the soil environment responds to combined long-term management is unclear.

The aim of the current study was to determine the effect of long-term Pi fertilisation on soil properties and specifically bacterial communities in two adjacent grassland soils which differed in terms of aboveground management (grazed vs. cut and removed). The grazed site (G) was rotationally grazed by cattle, whilst the cut and removed site (C) had the aboveground vegetation removed. Both sites had a long history of defined Pi fertilisation, receiving one of the following applications of calcium superphosphate: unfertilised (P0), 15 (P15) or 30 (P30) kg $ha^{-1}$ $yr^{-1}$. At the time of sampling, the grazed trial had been established for 45 years, and the cut trial for 18 years. Soil physicochemical properties, microbial biomass, phosphomonoesterase activity, bacterial community structure and bacterial *phoD* copy numbers were examined at both sites to determine if Pi fertilisation rate would increase soil P (Pi and total P), microbial biomass and potential P cycling ability, as well as harbour different bacterial community structures in a similar manner under both aboveground regimes.

## 2. Materials and Methods

### 2.1 Site descriptions

Both grassland field sites are located in Co. Wexford, Ireland: grazed 52°16'N, 06°30'W (Tunney et al., 2010) and cut 52°17'N, 06°30'W (Daly and Casey, 2006).

*Grazed site* (G) - A detailed description of the grazed site has been previously published (Culleton et al., 2002; King-Salter. 2008; Griffiths et al., 2012; Chen et al., 2014). Established in 1968 on a Humic Gleysol soil (IUSS WRB, 2015), the soil was initially sown with *Lolium perenne* and divided into thirty-six 0.45 ha plots, twelve per Pi fertilisation rate (G-P0, G-P15, G-P30 kg $ha^{-1}$ $yr^{-1}$), along with N (ammonium nitrate 250 kg $ha^{-1}$ $yr^{-1}$) and K (potassium chloride 20 kg $ha^{-1}$). After 1999, a consistent grazing stocking rate was applied (3,300 kg stock $ha^{-1}$) and Pi fertilisation rates were altered to assess recovery responses to Pi fertilisation (SI Fig 1). No legacy effect due to previous variation in stocking rates was detected across the site (Tunney et al., 2010). For the purpose of this study, only plots receiving continual Pi applications of 0 (G-P0), 15 (G-P15) or 30 (G-P30) kg Pi $ha^{-1}$ $yr^{-1}$ since 1968 were sampled (SI Fig 1).

*Cut and removed site* (C) - The cut site was established in February 1995 on a loam textured soil and divided into sixteen plots (10 x 2 m) in a fully randomised block design (Massey et al., 2012) (SI Fig 2). Each Pi treatment had four replicate plots receiving 16% superphosphate at fertilisation rates of 0 (C-P0), 15 (C-P15), 30 (C-P30) and 45 (P-45) kg Pi $ha^{-1}$ $yr^{-1}$ each February. Only plots receiving 0 (C-P0), 15 (C-P15) and 30 (C-P30) kg Pi $ha^{-1}$ $yr^{-1}$ were sampled to relate to the grazed site. Aboveground plant biomass at this site was cut eight times per year to a height of 5/6 cm using a plot harvester (Massey et al., 2015). All cuttings

were removed and sampled for dry matter yield and total plant P content. After each harvest, all plots received N (calcium ammonium nitrate, 40 kg N $ha^{-1}$) and K (potassium chloride, 125 kg K $ha^{-1}$ $yr^{-1}$), at rates to compensate for the removal of these nutrients by harvesting.

**2.2 Sample collection**

In October 2013, three plots per Pi fertilisation treatment (P0, P15 or P30) were sampled at both sites. For each of the eighteen plots sampled, a composite sample was made using a Dutch auger (4 cm diameter) by sieving (<2 mm). Sub-samples were stored at -20 $^{o}$C for molecular analysis, with the remainder stored at 4 $^{o}$C and processed for biochemical and physicochemical characterisation.

Total soil C and N were determined using an elemental analyser (LECO TrueSpec CN elemental analyser, US) from 0.2 g of ball milled soil dried at 40 $^{o}$C for 48 h as described by Griffiths et al., (2012). Total soil P was determined using microwave digestion (EPA, 2007) and measured via inductively coupled plasma (ICP) optical emission spectroscopy from 0.5 g of ball milled soil dried at 40 $^{o}$C for 48 h. Available Pi was determined using the Morgan's extraction method (McCormack, 2002), as described by Massey et al., (2012) and measured spectrophotometrically at 880 nm using the phosphomolybdate method (Murphy and Riley, 1962); inorganic N fractions within soil were determined by 2M potassium chloride extraction (Mulvaney, 1996) and analysed for $NO_3^-$ and $NH_4^+$ by colorimetric analysis using an Aquakem 600A (Konelab 60, US).

Soil pH was determined using a soil: deionised water ratio of 1:2 (w/v) (McCormack, 2002) measured using a WTW pH526 pH meter (Labsource, US); 5 g of soil was oven dried at 105 $^{o}$C for 24 h to determine the moisture content; soil water holding capacity (WHC) was

210 determined from 50 g of fresh soil placed into a Haines funnel with 100 ml deionised water
211 added to the soil and covered. After 24 h water was drained for 30 min and the volume
212 collected (Jenkinson and Powlson, 1976). Soil textural analysis was conducted following the
213 methodology by Culleton (1972).

214 **2.3 Microbial biomass**

215 Microbial biomass C, N (Murphy and Riley, 1962) and P (Brookes et al., 1982) were
216 measured using chloroform-fumigation extraction methods. Within the microbial biomass,
217 total organic carbon (TOC) was measured by combustion (Baird, 2005) using a Shimadzu
218 TOC-VCPH analyser with ASI-V autosampler; total organic nitrogen (TON) was determined by
219 alkaline persulfate oxidation (Cabrera and Beare, 1993) and available Pi by the ammonium
220 molybdate-ascorbic acid method (Watanabe and Olsen, 1965) using a Cary 50 Cone-UV
221 spectrophotometer. Soil microbial biomass C, N and P were calculated as the difference
222 between the fumigated and un-fumigated samples using conversion factors of 0.45 for C
223 (Wu et al., 1990), 0.45 for N (Jenkinson et al., 2004) and 0.40 for P (Hedley and Stewart,
224 1982). DOC and DON can both be calculated when determining the microbial biomass as the
225 amount of C and N in un-fumigated samples (Griffiths et al., 2012).

226 **2.4 Acid phosphomonoesterase activity**

227 Acid phosphomonoesterase (EC 3.13.2) activity was determined using a modified method of
228 Tabatabai and Bremner (1969), with toluene step removed. Optical density of the
229 supernatant was measured at 400 nm using a spectrophotometer (Helios Unicam).
230 Phosphomonoesterase activity was calculated with respect to standard curves constructed
231 using known concentrations of p-nitrophenol and expressed as μg PNP $g^{-1}$ DW soil $h^{-1}$.

232 **2.5 Soil DNA extraction**

233 DNA was extracted from 0.5 g of soil in triplicate per plot using a modified version of the
234 method published by Griffiths et al., (2000) (Storey et al., 2014). Extracts were quantified
235 and quality checked using a Nanodrop ND-1000 Spectrophotometer (ThermoFisher, UK) and
236 further cleaned using High Pure PCR product purification kit (Roche, UK) to ensure maximal
237 removal of PCR inhibitors.

238 **2.6 Amplicon sequencing of bacterial community**

239 Next generation sequencing of the bacterial *16S rRNA* gene was performed using a modified
240 protocol of Kozich et al., (2013). The forward (5'-
241 TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3') and reverse (5'-
242 AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3') primers which were previously ligated with
243 unique index combinations (Integrated DNA Technologies, USA): A501-A508 and A707, A708
244 and A709 were used for sequencing. Primers for PCR were reconstituted to 100 µM prior to
245 use. PCR was performed in 96 well plates containing 17 µl of Accuprime Pfx Supermix
246 (Invitrogen, UK), 1 µl target DNA, 2 µl of each primer set. 1 µl of PCR grade water was used
247 as a negative control in place of target DNA. PCR conditions consisted of a hot start of 95°C
248 for 2 min, followed by 95°C for 20 sec, 55°C for 15 sec and 72°C for 5 min (30 cycles) with a
249 final elongation of 72°C for 10 min. PCR products were visualised on a 1% (w/v) agarose gel
250 (Roche Diagnostics, Ireland). PCR products were cleaned and normalised using the
251 SequalPrep Normalization Plate kit (Invitrogen, UK) following manufacturer's instructions.
252 Samples were quantified using a Qubit™ 3.0 Fluorometer (ThermoFisher, UK) and quality
253 checked using a Nanodrop ND-1000 Spectrophotometer (ThermoFisher, UK). Sequencing
254 was carried out by the Centre for Genomic Research, University of Liverpool using an

255 Illumina MiSeq system and a V2 Illumina reagent kit. Raw sequences generated were
256 processed using the Mothur software (v.1.34) (Schloss et al., 2009). Illumina adapter
257 sequences were trimmed by cutadapt ver.2.1.1 using option -O 3. Quality filtering using
258 Sickle ver.1.2 further trimmed the data with a quality score of ≥20, followed by removal of
259 reads <10 bp after trimming. Each read was then trimmed to a maximum of 275 bp and
260 ambiguous bases were removed. Sequences which contained homopolymer runs >8 bases
261 were discarded. After trimming, identical sequences were grouped into 'unique' sequences.
262 Chimeric sequences were identified using the UCHIME algorithm within Mothur and were
263 then removed. From 2,395,612 contigs, 1,789,505 sequences were constructed and 419890
264 unique sequences established. The number of chimeric sequences removed equated to
265 122937. 195731 sequences were then assigned to operational taxonomic units (OTUs) using
266 the 'cluster' command and the average neighbour algorithm. All subsequent OTU-based
267 analyses were performed using a cut-off of 0.03. Taxonomy was assigned to the aligned
268 sequences by comparing processed data to the silva databases for bacteria (arb-
269 silva.de/silva-license-information). At the grazed site the average number of sequences per
270 sample equated to 27,020 ±3,465 standard errors of the mean. For the cut and removed
271 site, the average number of sequences per sample equated to 45,855 ±24,638 standard
272 errors of the mean. Rarefaction curves detailing the depth of sampling intensity showed
273 that for all treatments curves were well saturated (SI Fig 3). The final bacterial dataset was
274 then generated based on the relative abundances of bacterial genera. The average relative
275 abundance within each biological replicate was calculated according to the rules outlined by
276 Dunbar et al., (2001) i.e. a genus/genus-type had to be present in at least 2 technical
277 replicates (there were 3 technical replicates run for each biological replicate) to be
278 considered. The relative abundance was first averaged across the technical replicates and

this data was used to calculate relative abundance in each biological replicate. To account for differences in the number of sequencing reads between samples, samples were normalised to the biological sample containing the lowest number of reads prior to analysis. Bacterial genera/genus-type with relative abundances contributing to >0.1% of the total abundance were included in the dataset. Sequence files associated with each sample have been submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena), under accession number PRJEB21592.

**2.7 Quantification of bacterial *phoD* gene**

Real-time PCR (qPCR) was used to quantify copy numbers of the bacterial *phoD* gene from a standard curve constructed using plasmid from *Pseudomonas aeruginosa* PA0 (Fraser et al., 2015), using primer set ALPS-F730 (5'-CAGTGGGACGACCACGA GGT-3') and ALPS-R1101 (5'-GAGGCCGATCGGCATGTCG-3') (Sigma, UK) (Sakurai et al., 2008). After running a serial dilution on a sub-set of samples to test for inhibition, the *phoD* assay was performed (Fraser et al., 2015). Each sample was run in triplicate, alongside standards and negative controls on a ViiA7 qPCR instrument (Life Technologies, Ireland), in MicroAmp® optical 96-well reaction plates. Each reaction contained 6.25 µL of 2 X KAPA SYBR® FAST qPCR master mix, 0.25 µL ROX™ low passive reference dye (Anachem, Bedfordshire, UK), with 0.2 pmoles each primer and 2 ng of DNA using software ViiA RUO Ver 1.2.4 Applied Biosystems software (Life Technologies, Ireland) (Storey et al., 2014). The efficiency of the reaction, determined from the standard curve equated to 84% ($R^2$ = 0.997). The melt curve data confirmed no non-specific amplification and no amplification in the negative controls.

300 **2.8 Statistical analyses**

301 All univariate data was analysed using Minitab v15 (Minitab Ltd). Residuals of the data were
302 assessed for normal distribution using the Anderson-Darling normality test and Levene's test
303 for homogeneity of variances as assumptions to be satisfied before performing parametric
304 analyses. One-way ANOVAs were performed to test for an effect of Pi fertilisation rate
305 across each site separately on soil physicochemical and microbial biomass data, bacterial
306 richness scores and changes in relative abundance for the top 24 most abundant bacterial
307 genera, bacterial *phoD* copy numbers and acid phosphomonoesterase activity.

308 Multivariate statistics were used to analyse bacterial community structure using the
309 computer programme PRIMER-E version 6.1.9 with the PERMANOVA add-on version 1.0.1.
310 The raw relative abundance data were transformed to the $4^{th}$ root to down-weigh highly
311 abundant genera whilst recognising those at lower relative abundance (Clarke and Warwick,
312 2001). The similarity in community structure was determined by calculating Bray-Curtis
313 coefficients between samples which generated a resemblance matrix for comparisons
314 between complex multivariate biological samples to be made (Clarke et al., 2006). Bacterial
315 communities were classified by site (grazed: G or cut: C) and further distinguished by Pi
316 fertilisation rate (G-P0, G-P15, G-P30, C-P0, C-P15, C-P30). The bacterial dataset was tested
317 for homogeneity of variances at the lowest level of factor combinations using the PermDisp
318 function in PRIMER-E prior to further analysis.

319 The bacterial community structure for each Pi fertilisation treatment at both sites was
320 visualised using Non-metric Multidimensional Scaling (NMDS). Differences between
321 community structures exposed to different Pi fertilisation rates were tested by applying
322 statistical permutation-based multivariate analysis of variance tests (PERMANOVA,
323 Anderson, 2001), on the Bray-Curtis resemblance matrix and subsequent pair-wise

comparisons (Anderson et al., 2008). For the PERMANOVA test 9,999 permutations of the data were performed. Correlations between measured soil parameters were conducted to identify and remove covariates ($R^2$>0.7) (SI Table 1). This left 11 soil parameters: pH, moisture content (MC), soil C:P, soil C:N, DOC, Pi, $NH_4^+$, microbial biomass C:N, N:P and C:P. BIO-ENV tests were performed to select parameters explaining a significant proportion of variation within the bacterial dataset (Clarke and Ainsworth, 1993; Anderson et al., 2008; Wakelin et al., 2012).

## 3. Results

### 3.1 Soil physicochemical properties

Soil physicochemical properties are shown in Table 1. Soil pH ranged from 5.39-5.77 at the grazed site and from 5.64-5.91 at the cut site. Clay content (F=13.55, p=0.016), soil pH (F=7.84, p=0.019) and water holding capacity (F=13.72, p=0.014) were significantly higher at the cut site compared to the grazed site. Under grazing, total soil P and Pi increased significantly with Pi fertilisation, with Pi concentrations increasing from 3.13 mg $kg^{-1}$ DW soil in unfertilised (G-P0) plots to 20.83 mg $kg^{-1}$ DW soil in the G-P30 soil (F=7.56, p=0.042). Total P significantly increased (F=12.46, p=0.019) in both fertilised plots (G-P15 and G-P30) compared to unfertilised (G-P0) soil (Table 1). Total soil C and N were unresponsive to long-term Pi fertilisation rates, along with DOC, DON and inorganic N ($NO_3^-$/$NH_4^+$) (Table 1). A strong correlation was found between a number of the variables ($R^2$>0.7 SI Table 1).

Long-term differences in Pi fertilisation at the cut and removed site resulted in a significantly higher concentration of Pi in C-P30 soil at 4.32 mg $kg^{-1}$ DW soil compared to the unfertilised plot (C-P0) at 3.08 mg $kg^{-1}$ DW soil and C-P15 soil at 2.67 mg $kg^{-1}$ DW soil (Table

346 1). No other soil physicochemical parameter significantly differed across the cut and
347 removed site (Table 1).

348 **3.2 Soil microbial biomass C, N and P**

349 Soil microbial biomass P and C increased with Pi fertilisation rate under grazing, and
350 although a similar trend was observed for N, this was not significant (Fig 1). Microbial
351 biomass P increased significantly with Pi fertilisation rate from 98.05 mg $kg^{-1}$ DW soil in
352 unfertilised (G-P0) soil to 182.10 mg $kg^{-1}$ DW soil in G-P30 fertilised soil ($F=7.43$, $p=0.024$)
353 (Fig 1a), mirroring the response of soil Pi (Table 1). Microbial biomass C (Fig 1b) mirrored the
354 response of total soil P (Table 1), increasing significantly from 465.2 mg $kg^{-1}$ DW soil in
355 unfertilised (G-P0) soil to 1,386.5 mg $kg^{-1}$ DW soil in G-P15 and 1,484.6 mg $kg^{-1}$ DW soil in G-
356 P30 fertilised soil ($F=13.17$, $p=0.006$), but not significantly between G-P15 and G-P30
357 treatments (Fig 1b). At the cut and removed site, despite a significant difference in soil Pi
358 concentrations between C-P0 and C-P30 (Table 1), microbial biomass C, P and N remained
359 constant across the three Pi treatments (Fig 1).

360 **3.3 Acid phosphomonoesterase activity**

361 At the grazed site, phosphomonoesterase activity was significantly ($F=6.44$, $p=0.032$) higher
362 in unfertilised (G-P0) soil compared to fertilised soil (G-P15 and G-P30 (Fig 2). A similar trend
363 was observed at the cut and removed site, with activity significantly lower in the C-P30 soil
364 (199.99 µg PNP $g^{-1}$ DW soil $h^{-1}$) and C-P15 or C-P0 soils (210.48 and 257.09 µg PNP $g^{-1}$ DW
365 soil $h^{-1}$) compared to unfertilised (C-P0) soil (Fig 2).

### 3.3 Bacterial richness and community structure in soil exposed to long-term differences in Pi fertilisation rates

Across both the grazed and cut grassland sites, long-term differences in the Pi fertilisation rates did not result in significant differences in the richness of the bacterial communities (Table 2). However, the NMDS plot suggested differences in bacterial community structures at the grazed site exposed to different long-term Pi fertilisation, whereas no clear clustering appeared evident at the cut site (Fig 3).

PERMANOVA analysis confirmed bacterial community structure differed significantly ($p<0.05$) in soil exposed to different Pi fertilisation rates at the grazed site. Pairwise testing confirmed a significant ($p<0.001$) difference in bacterial community structure between G-P30 and unfertilised (G-P0) soil, with a proportion of the variation significantly explained by soil C:N and C:P (Rho=0.6, p=0.03).

Across the cut and removed grassland site, no significant differences were detected in bacterial community structure in response to long-term Pi fertilisation. The BIO-ENV test did not identify any soil parameters explaining a significant proportion of the variation within the bacterial dataset at this site.

### 3.4 Response of individual bacterial genera to long-term Pi fertilisation across grazed and cut and removed grassland sites

Amplicon sequencing revealed Acidobacteria to be the most relatively abundant phylum, with *Gp6* and *Gp16* both present within the top 3 most abundant genera at both sites (Table 3a and b). Significant differences in the relative abundances of the top 24 most abundant bacterial genera were detected between Pi fertilisation treatments at the grazed site, but not at the cut and removed site. Under grazing, the relative abundance of *Bradyrhizobium* (F=250.24,

p=0.001) from the *α-Proteobacteria* was significantly higher in unfertilised (G-P0) soil compared to G-P15 and G-P30 soil. An unclassified genus-type group belonging to *Actinobacteria* (F=5.73, p=0.041) along with another unclassified genus-type group this time belonging to *Acidimicrobiales* (F=8.74, p=0.017) significantly decreased between unfertilised and P30 plots only. In contrast, the relative abundance of bacterial classes belonging to the phylum Acidobacteria showed positive responses to Pi fertilisation: *Gp6* (F=14.84, p=0.005) were relatively more abundant in fertilised (G-P15 and G-P30) compared to unfertilised (G-P0) soil, while *Gp16* (F=10.51, p=0.011) responded positively to higher Pi fertilisation (G-P30) compared to unfertilised (G-P0) soil and G-P15 soil (Table 3a).

**3.5 Copy numbers of the bacterial phosphorus mineralising gene *phoD***

Under grazing, average copy numbers of the *phoD* gene increased significantly (F=6.03, p=0.037) from 5.1 x $10^3$ $g^{-1}$ DW soil in unfertilised (G-P0) plots to 4.0 x $10^4$ $g^{-1}$ DW soil in G-P15 and 4.2 x $10^4$ $g^{-1}$ DW soil in G-P30 treatments, but did not differ significantly between G-P15 and G-P30 plots (Fig 4).

At the cut and removed site, copy numbers were significantly (F=49.91, p=0.001) higher in C-P30 plots at 4.7 x $10^4$ $g^{-1}$ DW soil compared to unfertilised (C-P0) at 2.4 x $10^4$ $g^{-1}$ DW soil and C-P15 plots at 1.3 x $10^4$ $g^{-1}$ DW soil (Fig 4).

## 4. Discussion

As expected, the long-term addition of calcium superphosphate increased soil Pi concentrations at both sites (Griffiths et al., 2012; Massey et al., 2012). The extent of the effect however, differed. Concentrations of Pi, although significantly higher in C-P30 plots at the cut and removed site, were markedly lower than corresponding plots at the grazed site. The insensitivity of soil microbial biomass at the cut and removed site is likely a consequence of the lower availability of labile nutrients (i.e. $NO_2$/$NO_3^-$, DOC and Pi) generally observed at this site compared to the grazed. Reasons for the differences between sites however cannot be attributed solely to the difference in management as they are confounded by differences in soil properties and specifically, a higher soil pH and clay content at the cut site. Clay is particularly important as it can increase the capacity of phosphate ions to bind to soil particles, reducing Pi availability and also stabilise microbial biomass (Müller and Höper, 2004). Luckily, within each site confounding soil factors were not an issue so the effect of Pi fertilisation rates on key soil parameters within site could be assessed.

The effect of long-term differences in Pi fertilisation rate can contribute to differences in vegetation composition (Cruz et al., 2009). Previous work at both sites supports this. At the grazed site, unfertilised (G-P0) plots were dominated by poorer grasses: Common Bent (*Agrostis capillaris*) and Yorkshire fog (*Holcus lanatas*), as opposed to perennial ryegrass (*Lolium perenne*), cocksfoot (*Dactylis glomerata*), meadow grasses (*Poa* spp.) and white clover (*Trifolium repens*) (King-Salter, 2008; Tunney et al., 2010). At the cut and removed site, unfertilised (C-P0) plots were dominated by Bentgrass (*Agrostis genus*) whereas perennial ryegrass (*Lolium perenne*) dominated the fertilised (C-P15 and C-P30) plots (Massey et al., 2015). Whilst we cannot untangle the influences of vegetation

composition on the soil environment, here we consider it a direct result of the long-term Pi treatments.

***Bacterial community responses across a Pi fertilisation gradient in grazed and cut and removed grassland soils***

The effects of land-use on soil microbiota have been studied across a range of soil environments including bare fallow, bog, forest and arable (Jangid et al., 2008; Keith et al., 2012; Shange et al., 2012; Neal et al., 2017), but focus has mainly been on differences between extremes and location, rather than agricultural land-use (Keith et al., 2012; Tardy et al., 2015). Studies that have included fertilisation generally compare inorganic vs. organic fertilisers, or combined N and P fertilisation vs. unfertilised controls (Lemanski et al., 2014). The current study demonstrates that along a long-term Pi fertilisation gradient, distinctions in bacterial community structures typically occur between unfertilised and Pi fertilised soil, but also only when vegetation is grazed rather than cut and removed.

Despite the changes in bacterial community structure at the grazed site, no change in richness was detected at either site. Microbial richness responses to land-use have been varied (Keith et al., 2012; Tardy et al., 2015), suggesting it may not be an appropriate indicator of microbial responses to agricultural management.

The top 24 most abundant bacterial genera were similar at the two grassland sites. The overall acidity of the soils and similar long-term Pi fertilisation at both sites may have facilitated the establishment of similar bacterial communities over time (Jangid et al., 2008). The profiles of the soil bacteria are also consistent with other agricultural and grassland studies (Jangid et al., 2008; Nacke et al., 2011; Mander et al., 2012; Tan et al., 2013; Luo et al., 2015) suggesting a core bacterial microbiome. An unclassified genus-type belonging to

460 the order *Rhizobiales* was among the top 24 most relatively abundant genera at both sites;
461 members of this order have been shown to harbour *phoD* (Ragot et al., 2016) and other P
462 cycling genes (Bergkemper et al., 2016a). Significant differences in the individual relative
463 abundances were however only detected under grazing: *Bradyrhizobium* - a common
464 rhizosphere dwelling genus significantly decreased with Pi fertilisation between unfertilised
465 (G-P0) and fertilised (G-P15 and G-P30) grazed soil. Although *Bradyrhizobium* is typically
466 associated with N-fixation, higher relative abundances have been observed at low soil Pi
467 concentrations and linked to improved plant performance (Tairo and Ndakidemi, 2013;
468 Nyoki and Ndakidemi. 2014), and P mobilisation (Mander et al., 2012; Wakelin et al., 2012).
469 One possible explanation is that N fixing organisms such as *Bradyrhizobium* invest N for
470 production of N rich phosphatase enzymes (such as involved in P mineralisation) when soil P
471 concentrations are low (Nastro et al. 2014). An unclassified genus-type belonging to
472 *Actinobacteria* also responded negatively to Pi fertilisation, but in G-P30 plots only. The
473 phylum Actinobacteria has been closely linked with P solubilisation in soil (Mander et al.,
474 2012) and previously reported to respond negatively to Pi fertilisation (Wakelin et al., 2012).
475 Conversely, the relative abundance of genera belonging to the phylum Acidobacteria
476 responded positively to Pi fertilisation under grazing. Acidobacteria are estimated to
477 comprise 20% of bacteria within grassland soils (Quaiser et al., 2003), dominated by the
478 genera *Gp6* (Naether et al., 2012), *Gp4, Gp16* and *Gp7* (Will et al., 2010) as observed in this
479 study. Acidobacteria have been suggested to be associated with oligotrophic environments
480 due to their relatively slow growth (Jangid et al., 2008), however continual detection across
481 a range of agricultural soils suggests ecological roles currently not understood (Nunes da
482 Rocha et al., 2014). Previous work at the grazed site used in the current study reported a
483 negative response of the Acidobacteria to Pi fertilisation (Tan et al., 2013). This discrepancy

484 may reflect the different sequencing approaches (Roche 454 pyrosequencing vs. Illumina),
485 and time of year samples were collected however.

486 Soils are dynamic systems which respond to changes in the surrounding
487 environment. Seasonality is a key moderator of substrate availability, microbial activity and
488 can shape community structures (Dumbrell et al., 2011; Sorensen et al., 2013 Slaughter et
489 al., 2015). For example, seasonal effects were previously detected at the cut and removed
490 site on soil Pi, microbial biomass C and PLFA profiles (Massey et al., 2015). It should be
491 noted however that while Pi concentrations changed in the abovementioned study, they
492 remained relatively low. Nonetheless, this highlights the need to undertake longer-term
493 studies sampled across seasons, using techniques with similar levels of discrimination and
494 coverage before we can inform future strategies for sustainable agriculture.

495

496 ***Insights into P cycling across a Pi fertilisation gradient in grazed and cut and removed***
497 ***grassland soils***

498 The capacity for P mineralisation is a relatively broad function within soil microorganisms
499 (Ragot et al., 2016), with genetic similarity in P mineralising genes observed across
500 agricultural soils (Neal et al., 2017). One of the key enzymes involved in P mineralisation,
501 phosphomonoesterase's activity typically decreases as Pi availability increases (Olander and
502 Vitousek, 2000; Shi et al., 2013; Turner and Wright, 2014) and similar results were observed
503 at both sites in the current study. The decrease in activity in soils with higher Pi
504 concentrations most likely reflects more efficient microbial P uptake systems in P-depleted
505 soil (Bergkemper et al., 2016a).

506 In contrast, copy numbers of *phoD* increased with Pi fertilisation and numbers were
507 relatively similar between sites. The abundance of this gene has been shown to respond in a

similar manner to Pi fertilisation in other studies (Keith et al., 2012; Chhabra et al., 2013; Tan et al., 2013; Fraser et al., 2015; Ragot et al., 2016). Although *phoD* is considered the most abundant monophosphatase gene within soil and marine environments (Luo et al., 2009; Neal et al., 2017) and has been used as a proxy for bacterial P mineralisation (Tan et al., 2013; Fraser et al., 2015; Acuña et al., 2016; Ragot et al., 2016), it only provides partial insight into microbial P mineralisation. The inclusion of additional P mineralising genes such as *phoA* and *phoX* (Ragot et al.et al., 2016) and a *phoD* primer set offering better coverage (Bergkemper et al., 2016b) might have provided greater overview of this process. A metatranscriptomic or a metaproteomic analysis targeting additional microbial P cycling pathways would afford even greater insight.

The different response of a P cycling enzyme and the abundance of a gene encoding a phosphatase likely reflects the fact that gene abundance is merely a measure of the specific P cycling potential of the bacterial community, whereas the enzyme represents a direct measure of more general microbial activity and/or the absence of a direct relationship between the enzyme and gene measured. Despite differences in microbial biomass and soil Pi concentrations between the grazed and cut sites, bacterial *phoD* copy numbers and phosphomonoesterase activity were relatively similar in both sites. This suggests bacterial *phoD* copy numbers were not solely related to microbial biomass, and that P mineralisation may be an important mode of Pi mobilisation within reduced input systems. In soils where organic matter inputs are reduced i.e. bare fallow, a dependence on P mineralising exoenzymes has been detected, alongside increased phytate mineralisation (a recalcitrant source of organic P) (Neal et al., 2017). Copy numbers of β-propeller phytases involved in phytate mineralisation have also been documented to increase in reduced input

systems (Neal et al., 2017), and when soil Pi concentrations are low (Jorquera et al., 2013). It would be interesting to explore this further across our two sites.

## Conclusions

At the grazed site, increasing long-term Pi fertilisation rates resulted in increased soil P concentrations, increased microbial biomass C and P, significant shifts in bacterial community structures and differences in the relative abundances of some bacterial genera. In contrast at the cut and removed site, microbial biomass (C, N and P) and bacterial community structures did not respond to long-term increases in Pi fertilisation and soil Pi concentrations only increased under the highest fertilisation rate (C-P30). Despite differences in soil P concentrations and microbial biomass between the two sites, the abundance of *phoD* increased with increasing soil Pi across both sites, while phosphomonoesterase activity decreased. The current study demonstrates the potential impacts intensive cutting and removal of vegetation may impose on Pi fertiliser effectiveness, both in terms of P accumulation and the bacterial community. The identification of bacterial genera that respond to different rates of Pi fertilisation in grazed soil provides the opportunity to further examine the role of these genera within terrestrial P cycling.

## Acknowledgements

This research was conducted as part of the Earth and Natural Sciences Doctoral Studies Programme, funded under the Programme for Research in Third-Level Institutions and co-funded under the European Regional Development Fund. We are grateful to technical staff at Teagasc Environmental Research Centre. We are also thankful to John Murphy and additional members of Teagasc staff who helped establish and maintain the field trials used in the study. Work at SRUC was funded by the Scottish Government, Rural and Environment,

Science and Analytical Services Division. The authors would also like to acknowledge the knowledge provided by Dr John McGrath (Queen's University Belfast) concerning microbial P cycling, along with Dr Jim Grant (Teagasc, Ireland) and Dr Robert Ferguson (University of Essex) for their input on the statistical approach for the data, and Dr Corinne Whitby (University Essex) for editing advice. The authors do not consider there to be any conflict of interest concerning this research.

591 **References**

592 Abdalla, M., Hastings, A., Chadwick, D.R., Jones, D.L., Evans, C.D., Jones, M.B., Rees, R.M.,
593 Smith, P., 2018. Critical review of the impacts of grazing intensity on soil organic carbon
594 storage and other soil quality indicators in extensively managed grasslands. Agric. Ecosyst.
595 Environ. 253, 62-81.

596 Acuña, J.J., Durán, P., Lagos, L.M., Ogram, A., de la Luz Mora, M., Jorquera, M.A., 2016.
597 Bacterial alkaline phosphomonoesterase in the rhizospheres of plants grown in Chilean
598 extreme environments. Biol. Fert. Soils. 52, 763-773.

599 Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA+ for PRIMER: Guide to
600 software and statistical methods. Plymouth, UK.

601 Anderson, M., 2001. A new method for non-parametric multivariate analysis of variance.
602 Austral. Ecol. 26, 32–46.

603 Baird, R.B., 2005. Aggregate organic constituents; total organic carbon (TOC)/method 5310
604 B high temperature combustion method. In: Eaton, D.A., Classer, L.S., Rice, E.W.,
605 Greensburg, A.E., eds. Standard methods for the examination of waters and waste water.
606 21st ed. Washington DC: American Public Health Association.

607 Beauregard, M.S., Hamel, C., Atul-Nayyar, St-Arnaud, M., 2010. Long-term phosphorus
608 fertilization impacts soil fungal and bacterial diversity but not AM fungal community in
609 alfalfa. Microb. Ecol. 59, 379–389.

610 Bergkemper, F., Schöler, A., Engel, M., Lang, F., Krüger, J., Schloter, Schulz, S., 2016a.
611 Phosphorus depletion in forest soils shapes bacterial communities towards phosphorus
612 recycling systems. Environ Microbiol. 18, 1988-2000.

Bergkemper, F., Kublik, S., Lang, F., Krüger, J., Vestergaard, G., Schloter M., Schulz, S., 2016b. Novel oligonucleotide primers reveal a high diversity of microbes which drive phosphorous turnover in soil. J. Microbiological Meth. 125, 91-97.

Brookes, P.C., Powlson, D.S., Jenkinson, D.S., 1982. Measurement of microbial biomass phosphorus in soil. Soil Biol. Biochem. 14, 319–329.

Cabrera, M.L., and Beare, M.H., 1993. Alkaline persulfate oxidation for determining total nitrogen in microbial biomass extracts. Soil Sci. Soc. Am. J. 57, 1007-1012. DOI:10.2136/sssaj1993.03615995005700040021x

Chhabra, S., Brazil, D., Morrissey, J., Burke, J., O'Gara, F., Dowling, D., 2013. Fertilization management affects the alkaline phosphatase bacterial community in barley rhizosphere soil. Biol. Fertil. Soils 49, 31–39.

Chen, X., Daniell, T.J., Neilson, R., O'Flaherty, V., Griffiths, B.S., 2014. Microbial and microfaunal communities in phosphorus limited, grazed grassland change composition but maintain homeostatic nutrient stoichiometry. Soil Biol. Biochem. 75, 94–101.

Clarke, K.R., Somerfield, P.J., Chapman, M.G., 2006. On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray-Curtis coefficient for denuded assemblages. J. Exp. Mar. Bio. Ecol. 330, 55–80.

Clarke, K. R., and Warwick, R.M., 2001. Change in marine communities: an approach to statistical analysis and interpretation. Primer-E Ltd. Plymouth, UK.

Clarke, K.R., and Ainsworth, M., 1993. A method of linking multivariate community structure to environmental variables. Mar. Ecol. Prog. Ser. 92, 205-219.

COM, 91/676/EEC of the Council of December 12, 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources European Commission, Brussels.

637 COM, 2000/60/EC of the European Parliament and of the Council of 23 October 2000
638 establishing a framework for Community action in the field of water policy. Commission of
639 the European Communities. Brussels.
640 Cruz, A., Hamel, C., Hanson, K., Selles, F., Zentner, R.P., 2009. Thirty-seven years of soil
641 nitrogen and phosphorus fertility management shapes the structure and function of the soil
642 microbial community in a Brown Chernozem. Plant Soil. 315, 173-184.
643 Culleton, E., 1972. Laboratory analyses in soil survey investigations: theory and techniques.
644 An Foras Talúntais National Soil Survey, Johnstown Castle, Wexford, Republic of Ireland.
645 Culleton, N., Coulter, B., Liebhardt, W.C., 2002. The fate of phosphatic fertiliser applied to
646 grassland. Ir. Geogr. 35, 175–184.
647 Daly, K., and Casey, A., 2006. Environmental aspects of soil phosphorus testing. Ir. J. Agric.
648 Food Res. 44, 261-279.
649 de Vries, F. T., Hoffland, E., van Eekeren, N., Brussard, L., Bloem, J., 2006. Fungal/Bacterial
650 ratios in grasslands with contrasting nitrogen management. Soil Biol. Biochem. 38, 2092-
651 2103.
652 Dotaniya, M.L., and Meena, V.D., 2014. Rhizosphere effect on nutrient availability in soil and
653 its uptake by plants: A review. Proc. Natl. Acad. Sci. India Sect. B (Biol. Sci). 85, 1–12.
654 Dumbrell, A.J., Ashton, P.D., Aziz, N., Feng, G., Nelson, M., Dytham, C., Fitter, A.H., Helgason,
655 T., 2011. Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by
656 massively parallel pyrosequencing. New Phytol. 190, 794–804.
657 Dunbar, J., Ticknor, L.O., Kuske, C.R., 2001. Phylogenetic specificity and reproducibility and
658 new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from
659 bacterial communities. Appl. Environ. Microbiol. 67, 190–197.

EU, 2014. Good agricultural practice for protection of waters regulations. Statutory Instruments No. 31 of 2014. 1-50. Available online: https://www.agriculture.gov.ie/media/migration/ruralenvironment/environment/nitrates/SI31of2014290114.pdf [Accessed 13/11/2015]

FAO, 2015. Soil is a non-renewable resource. Available online: http://www.fao.org/fileadmin/user_upload/soils2015/docs/EN/IYS_fact_sheets_preservation_en_PRINT.pdf. [Accessed 01/04/2015].

FAO, 2010. Challenges and opportunities for carbon sequestration in grassland systems: A Technical Report on Grassland Management and Climate Change Mitigation, Integrated Crop Management, 9, 1-67. Available online: http://www.fao.org/fileadmin/templates/agphome/documents/climate/AGPC_grassland_webversion_19.pdf [Accessed 11/02/2016]

Fan, H., Wu, J., Liu, W., Yuan, Y., Hu, L., Cai, W., 2015. Linkages of plant and soil C:N:P stoichiometry and their relationships to forest growth in subtropical plantations. Plant Soil. 392, 127-138.

Fitter, A.H., Graves, J.D., Wolfenden, J., Self, G.K., Brown, T.K., Bogie, D., Mansfield, T.A., 1997. Root production and turnover and carbon budgets of two contrasting grasslands under ambient and elevated atmospheric carbon dioxide concentrations. New Phytol. 137, 47–255.

Fraser, T., Lynch, D.H., Entz, M.H., Dunfield, K.E., 2015. Linking alkaline phosphatase activity with bacterial phoD gene abundance in soil from a long-term management trial. Geoderma. 257–258, 115–122.

682 Griffiths, B.S., Spilles, A., Bonkowski, M., 2012. C:N:P stoichiometry and nutrient limitation
683 of the soil microbial biomass in a grazed grassland site under experimental P limitation or
684 excess. Ecol. Process. 1, 1–11.
685 Griffiths, R.I., Whiteley, A.S., Anthony, G., Donnell, O., Bailey, M.J., Donnell, A.G.O., 2000.
686 Rapid method for coextraction of DNA and RNA from natural environments for analysis of
687 ribosomal DNA- and rRNA-based microbial community composition. Appl. Environ.
688 Microbiol. 66, 5488–5491.
689 Gorazda, K., Wzorek, Z., Tarko, B., Nowak, A.K., Kulczycka, J., Henclik, A., 2013. Phosphorus
690 cycle - possibilities for its rebuilding. Acta Biochim. Pol. 60, 725–730.
691 Hedley, M.J., and Stewart, J.W.B., 1982. Method to measure microbial phosphate in soils.
692 Soil Biol. Biochem. 14, 377–385.
693 Hungate, B.A., Holland, E.A., Jackson, R.B., Chapin, F.S., Mooney, H.A., Field, C.B., 1997. The
694 fate of carbon in grasslands under carbon dioxide enrichment. Nature. 388, 576–579.
695 Isselstein, J., Jeangros, B., Pavlu, V., 2005. Agronomic aspects of biodiversity targeted
696 management of temperate grasslands in Europe – a review. Agron. Res. 3, 139–151.
697 Jackson, C.R., Roden, E.E., Churchill, P.F., 2000. Denaturing gradient gel electrophoresis can
698 fail to separate *16S rDNA* fragments with multiple base differences. Mol. Biol. Today. 1, 49-
699 51.
700 IUSS Working Group WRB. 2015. World Reference Base for Soil Resources 2014, update
701 2015 International soil classification system for naming soils and creating legends for soil
702 maps. World Soil Resources Reports No. 106. FAO, Rome. Available online:
703 http://www.fao.org/3/i3794en/I3794en.pdf [Accessed 15/10/2012]Jangid, K., Williams,
704 M.A., Franzluebbers, A.J., Sanderlin, J.S., Reeves, J.H., Jenkins, M.B., Endale, D.M., Coleman,
705 D.C., Whitman, W.B., 2008. Relative impacts of land-use, management intensity and

706 fertilization upon soil microbial community structure in agricultural systems. Soil Biol.
707 Biochem. 40, 1-11.
708 Jenkinson, D.S., Brookes, P.C., Powlson, D.S., 2004. Measuring soil microbial biomass. Soil
709 Biol. Biochem. 36, 5–7.
710 Jenkinson, D.S., and Powlson, D.S., 1976. The effects of biocidal treatments on metabolism
711 in soil—I. Fumigation with chloroform. Soil Biol. Biochem. 8, 167–177.
712 Jorquera, M.A., Martínez, O.A., Marileo, L.G., Acuña, J.J., Saggar, S., Mora, M.L., 2013.
713 Effect of nitrogen and phosphorus fertilization on the composition of rhizobacterial
714 communities of two Chilean Andisol pastures. World J. Microbiol Biotechnol. 30, 99–107.
715 Kageyama, H., Tripathi, K., Rai A., Cha-Um, S., Waditee-Sirisattha, R, Takabe, T., 2011. An
716 alkaline phosphatase/phosphodiesterase, *PhoD*, induced by salt stress and secreted out of
717 the cells of *Aphanothece halophytica*, a halotolerant cyanobacterium. Appl. Environ.
718 Microbiol. 77, 5178-5183.
719 Keith, A.M., Boots, B., Hazard, C., Niechoj, R., Arroyo, J., Bending, G.D., Bolger, T., Breen, J.,
720 Clipson, N., Doohan, F.M., Griffin, C.T., Schmidt, O., 2012. Cross-taxa congruence, indicators
721 and environmental gradients in soils under agricultural and extensive land management.
722 Eur. J. Soil Biol. 49, 55-62.
723 King-Salter, G.E., 2008. Response of arbuscular mycorrhizal fungi to seasonality and long-
724 term phosphorus fertilisation in an Irish grazed grassland. PhD Thesis, University College
725 Dublin, Dublin, Ireland.
726 Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of
727 a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence
728 data on the miseq illumina sequencing platform. Appl. Environ. Microbiol. 79, 5112–5120.

729 Lemanski, K., and Scheu, S., 2015. The influence of fertilizer addition, cutting frequency and
730 herbicide application on soil organisms in grassland. Biol. Fertil. Soils. 51, 197–205.
731 Li, J., Li, Z., Wang, F., Zou, B., Chen, Y., Zhao, J., Mo, Q., Li, Y., Li, X., Xia, H., 2015. Effects of
732 nitrogen and phosphorus addition on soil microbial community in a secondary tropical
733 forest of China. Biol. Fertil. Soils. 51, 207–215.
734 Liebisch, F., Keller, F., Huguenin-Elie, O., Frossard, E., Oberson, A., Bünemann, E.K., 2014.
735 Seasonal dynamics and turnover of microbial phosphorus in a permanent grassland. Biol.
736 Fertil. Soils. 50, 465–475.
737 Liu, L., Zhang, T., Gilliam, F.S., Gundersen, P., Zhang W., Chen, H., Mo, J., 2013. Interactive
738 effects of nitrogen and phosphorus on soil microbial communities in a tropical forest. PLoS
739 One. 8, doi.org/10.1371/journal.pone.0061188.
740 Luo, H., Benner, R., Long, R.A., Hu, J., 2009. Subcellular localization of marine bacterial
741 alkaline phosphatase. PNAS. 106, 21219–21223. DOI: 10.1073/pnas.0907586106.
742 Luo, P., Han, X., Wang, Y., Han, M., Shi, H., Liu, N., Bai, H., 2015. Influence of long-term
743 fertilization on soil microbial biomass, dehydrogenase activity, and bacterial and fungal
744 community structure in a brown soil of northeast China. Ann. Microbiol. 65, 533–542.
745 Mander, C., Wakelin, S., Young, S., Condron, L., O'Callaghan, M., 2012. Incidence and
746 diversity of phosphate-solubilising bacteria are linked to phosphorus status in grassland
747 soils. Soil Biol. Biochem. 44, 93–101.
748 Massey, P. A., 2012. Interactions between phosphorus fertilisation and soil biota in
749 managed grassland systems. PhD Thesis, Cranfield University, UK

750 Massey, P.A., Creamer, R.E., Whelan, M.J., Ritz, K., 2015. Insensitivity of soil biological
751 communities to phosphorus fertilization in intensively managed grassland systems. Grass
752 Forage Sci. 71, 139-152.
753 McCormack, S., 2002. Analysis of agricultural materials: *Methods used at Johnstown Castle*
754 *Research Centre*, Johnstown Castle Research Centre, Wexford, Ireland, vol. 2.
755 McSherry, M.E., and Ritchie, M.E., 2013. Effects of grazing on grassland soil carbon: a global
756 review. Global Change Biol. 19, 1347-1357.
757 Mulvaney, R.L., 1996. Nitrogen—inorganic forms. In: Sparks, D.L., Page, A.L., Helmke, P.A.,
758 Loeppert, R.H., Soltanpour, P.N., Tabatabai, M.A., Johnson, C.T. and Sumner, M.E., eds.,
759 Methods of soil analysis Part 3. Madison: SSSA. 1123-1184.
760 Müller, T., and Höper, H., 2004. Soil organic matter turnover as a function of the soil clay
761 content: Consequences for model applications. Soil Biol. Biochem. 36, 877–888.
762 Murphy, J., and Riley, J.P., 1962. A modified single solution method for the determination of
763 phosphate in natural waters. Anal. Chim. Acta. 27, 31–36.
764 Nacke, H., Thürmer, A., Wollherr, A., Will, C., Hodac, L., Herold, N., Schöning, I., Schrumpf,
765 M., Daniel, R., 2011. Pyrosequencing-based assessment of bacterial community structure
766 along different management types in German Forest and Grassland soils. PLoS One. 6, 1–12.
767 DOI: 10.1371/journal.pone.0017000.
768 Nasto, M.K., Alvarez-Clare, S., Lekberg, Y., Sullivan, B.W., Townsend, A.R., Cleveland, C.C.,
769 2014. Interactions among nitrogen fixation and soil phosphorus acquisition strategies in
770 lowland tropical rain forests. Ecol. Lett. 17, 1282–1289.
771 Naether, A., Foesel, B.U., Naegele, V., Wüst, P.K., Weinert, J., Bonkowski, M., Alt, F.,
772 Oelmann, Y., Polle, A., Lohaus, G., Gockel, S., Hemp, A., Kalko, E.K. V, Linsenmair, K.E.,
773 Pfeiffer, S., Renner, S., Schöning, I., Weisser, W.W., Wells, K., Fischer, M., Overmann, J.,

774 Friedrich, M.W., 2012. Environmental factors affect acidobacterial communities below the
775 subgroup level in grassland and forest soils. Appl. Environ. Microbiol. 78, 7398–7406.
776 Neal, A.L., Rossmann, M., Brearley, C., Akkari, E., Guyomar, C., Clark, I.M., Allen, E., Hirsch,
777 P.R., 2017. Land-use influences phosphatase gene microdiversity in soil. Environ Microbiol.
778 19, 2740-2753.
779 Nitschelm, J.J., Luscher, A., Hartwig, U.A., van Kessel, C., 1997. Using stable isotopes to
780 determine soil carbon input differences under ambient and elevated atmospheric $CO_2$
781 conditions. Global Change Biol. 3, 411–416.
782 Nunes da Rocha U, Plugge C.M., George, I., van Elsas, J.D, van Overbeek, L.S., 2013. The
783 rhizosphere selects for particular groups of Acidobacteria and Verrucomicrobia. PLoS One. 8.
784 DOI: 10.1371/journal.pone.0082443.
785 Nyoki, D., and Ndakidemi, P.A., 2014. Effects of *Bradyrhizobium japonicum* inoculation and
786 supplementation with phosphorus on macronutrients uptake in cowpea (*Vigna unguiculata*
787 (L.) Walp). Am J. Plant Sci. 5, 442–451.
788 O'Mara, F.P., 2012. The role of grasslands in food security and climate change. Ann. Bot.
789 110, 1263–1270.
790 Olander, L.P, and Vitousek P.M., 2000. Regulation of soil phosphatase and chitinase activity
791 by N and P availability. Biogeochemistry. 49, 175–190.
792 Osorio, N., and Habte, M., 2013. Phosphate desorption from the surface of soil mineral
793 particles by a phosphate-solubilizing fungus. Biol. Fertil. Soils. 49, 481–486.
794 Owen, D., Williams, A.P., Griffith, G.W., Withers, P.J.A., 2015. Use of commercial bio-
795 inoculants to increase agricultural production through improved phosphorous acquisition.
796 Appl. Soil Ecol. 86, 41–54.

797 Poeplau, C., Marstop, H., Thorez, K., Kätterer, T., 2016. Effect of grassland cutting frequency
798 on soil carbon storage – a case study on public lawns in three Swedish cities. SOIL. 2, 175-
799 184.
800 Philippot, L., Ritz, K., Pandard, P., Hallin, S., Martin-Laurent, F., 2012. Standardisation of
801 methods in soil microbiology: Progress and challenges. FEMS Microbiol. Ecol. 82, 1–10.
802 Quaiser, A., Ochsenreiter, T., Lanz, C., Schuster, S.C., Treusch, A.H., Eck, J., Schleper, C.,
803 2003. Acidobacteria form a coherent but highly diverse group within the bacterial domain:
804 evidence from environmental genomics. Mol. Microbiol. 50, 563–575.
805 Ragot, S.A., Kertesz, M.A., Bünemann, E.K., 2016. *phoD* alkaline phosphatase gene diversity
806 in soil. Appl. Environ. Microbiol. 81, 7281-7289.
807 Richardson, A.E., and Simpson, R.J., 2011. Soil microorganisms mediating phosphorus
808 availability update on Microbial Phosphorus. Plant Physiol. 156, 989–996.
809 Rumpel, C., Crème, A., Ngo, P.T., Velásquez, G., Mora, M.L., Chabbi, A., 2015. The impact of
810 grassland management on biogeochemical cycles involving carbon, nitrogen and
811 phosphorus. J. Soil Sci. Plant Nutr. 15, 353-371.
812 Sakurai, M., Wasaki, J., Tomizawa, Y., Shinano, T., Osaki, M., 2008. Analysis of bacterial
813 communities on alkaline phosphatase genes in soil supplied with organic matter. J. Soil Sci.
814 Plant Nutr. 54, 62–71.
815 Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski,
816 R. A. Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van
817 Horn, D. J., Weber, C. F., 2009. Introducing mothur: open-source, platform-independent,
818 community-supported software for describing and comparing microbial communities. Appl.
819 Environ. Microbiol. 75, 7537-7541.

Sharma, S.B., Sayyed, R.Z., Trivedi, M.H., Gobi, T.A., 2013. Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. Springerplus. 2, 1-14. DOI: 10.1186/2193-1801-2-587

Shi Y, Lalande R, Hamel C, Ziadi N, Gagnon B, Hu Z., 2013. Seasonal variation of microbial biomass, activity, and community structure in soil under different tillage and phosphorus management practices. Biol. Fertil. Soils. 49, 803–818.

Simpson, R.J., Oberson, A., Culvenor, R.A., Ryan, M.H., Veneklaas, E.J., Lambers, H., Lynch, J.P., Ryan, P.R., Delhaize, E., Smith, F.A., Smith, S.E., Harvey, P.R., Richardson, A.E., 2011. Strategies and agronomic interventions to improve the phosphorus-use efficiency of farming systems. Plant Soil. 349, 89-120.

Sims, J.T., and Sharpley, A.N., (eds) 2005. Phosphorus: Agriculture and the environment Agronomy monograph no. 46, American Society of Agronomy, Inc.; Crop Science Society of America, Inc.; Soil Science Society of America, Inc., United States of America.

Slaughter, L.C., Weintraub, M.N., McCulley, R.L., 2015. Seasonal effects stronger than three-year climate manipulation on grassland soil microbial community. Soil Sci. Soc. Am. J. 79.

Sorensen, P.O., Germino, M.J., Feris, K.P., 2013. Microbial community responses to 17 years of altered precipitation are seasonally dependent and coupled to co-varying effects of water content on vegetation and soil C. Soil Biol. Biochem. 64, 155–163.

Soussana, J.F., and Lemaire, G., 2014. Coupling carbon and nitrogen cycles for environmentally sustainable intensification of grasslands and crop-livestock systems. Agric. Ecosyst. Environ. 190, 9-17.

Storey, S., Ashaari, M.M., McCabe, G., Harty, M., Dempsey, R., Doyle, O., Clipson, N., Doyle, E.M., 2014. Microbial community structure during fluoranthene degradation in the presence of plants. J. Appl. Microbiol. 11, 74-84.

844 Stutter, M.I., 2015. The composition, leaching, and sorption behaviour of some alternative
845 sources of phosphorus for soils. Ambio. 44, 207-216. DOI 10.1007/s13280-014-0615-7.
846 Tabatabai, M.A., and Bremner, J.M., 1969. Use of p-nitrophenyl phosphate for assay of soil
847 phosphatase activity. Soil Biol. Biochem. 1, 301–307.
848 Tairo, E.V, and Ndakidemi, P.A., 2013. *Bradyrhizobium japonicum* inoculation and
849 phosphorus supplementation on growth and chlorophyll accumulation in Soybean (*Glycine*
850 *max* L.). Am J. Plant Sci. 4, 2281–2289.
851 Tan, H., Barret, M., Mooij, M., Rice, O., Morrissey, J., Dobson, A., Griffiths, B., O'Gara, F.,
852 2013. Long-term phosphorus fertilisation increased the diversity of the total bacterial
853 community and the *phoD* phosphorus mineraliser group in pasture soils. Biol. Fertil. Soils.
854 49, 661–672.
855 Tao, G., Tian, S., Cai, M., Xie, G., 2008. Phosphate-solubilizing and mineralizing abilities of
856 bacteria isolated from soils. Pedosphere. 18, 515-523.
857 Tardy, V., Spor, A., Mathieu, O., Leveque, J., Terrat, S., Plassart, P., Regnier, T., Bardgett,
858 R.D., van der Putten, W.M., Roggero, P.R., Seddaiu, G., Bagella, S., Lemanceau, P., Ranjard,
859 L., Maron, P., 2015. Shifts in microbial diversity through land use intensity as drivers of
860 carbon mineralization in soil. Soil Biol. Biochem. 90, 204-213.
861 Tilman, D., Cassmand, K.G., Matson, P.A., Naylor, R., Polasky, S., 2002. Agricultural
862 sustainability and intensive production practices. Nature. 418, 671-677.
863 Tunney, H., Kirwan, L., Fu, W., Culleton, N., Black, A.D., 2010. Long-term phosphorus
864 grassland experiment for beef production – impacts on soil phosphorus levels and
865 liveweight gains. Soil Use Manag. 26, 237–244.

866 Turner, B, and Wright, J.S., 2014. The response of microbial biomass and hydrolytic enzymes
867 to a decade of nitrogen, phosphorus, and potassium addition in a lowland tropical rain
868 forest. Biogeochemistry. 117, 115–130.
869 van Dorst, J., Bissett, A., Palmer, A.S., Brown, M., Snape, I., Stark, J.S., Raymond, B.,
870 McKinlay, J., Ji, M., Winsley, T., Ferrari, B.C., 2014. Community fingerprinting in a sequencing
871 world. FEMS Microbiol. Ecol. 89, 316-330.
872 Wakelin, S., Mander, C., Gerard, E., Jansa, J., Erb, A., Young, S., Condron, L., O'Callaghan, M.,
873 2012. Response of soil microbial communities to contrasted histories of phosphorus
874 fertilisation in pastures. Appl. Soil Ecol. 61, 40–48.
875 Watanabe, F.S., and Olsen, S.R., 1965. Test of an ascorbic acid method for determining
876 phosphorus in water and $NaHCO_3$ extracts from soil. Soil Sci. Soc. Am. J. 29, 677-678.
877 Will, C., Thümer, A., Wollherr, A., Nacke, H., Herold, N., Schrumpf, M., Gutknecht, J., Wubet,
878 T., Buscot, F., Daniel, R., 2010. Horizon-specific bacterial community composition of German
879 grassland soils, as revealed by pyrosequencing-based analysis of 16S rRNA genes. Appl.
880 Environ. Microbiol. 76, 6751-6759.
881 Wu, J., Joergensen, R.G., Pommerening, B., Chaussod, R., Brookes, P.C., 1990. Measurement
882 of soil microbial biomass C by fumigation-extraction—an automated procedure. Soil Biol.
883 Biochem. 22, 1167–1169.

**Table 1**

| | Pi fertilisation rate (kg $ha^{-1}$ $yr^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Grazed site | | | Cut and removed site | | |
| | **G-P0** | **G-P15** | **G-P30** | **C-P0** | **C-P15** | **C-P30** |
| Sand (%) | 51 *(2)* [a] | 44 *(5)* [a] | 53 *(2)* [a] | 47 *(1)* [A] | 45 *(1)* [A] | 46 *(1)* [A] |
| Silt (%) | 31 *(1)* [a] | 32 *(2)* [a] | 29 *(2)* [a] | 32 *(1)* [A] | 32 *(3)* [A] | 30 *(2)* [A] |
| Clay (%) | 18 *(3)* [b] | 17 *(1)* [b] | 18 *(1)* [b] | 21 *(1)* [A] | 23 *(2)* [A] | 24 *(2)* [A] |
| pH | 5.39 *(0.03)* [a] | 5.77 *(0.07)* [a] | 5.63 (*0.02*) [a] | 5.64 (*0.21*) [A] | 5.91 (*0.11*) [A] | 5.86 (*0.14*) [A] |
| Water holding capacity (%) | 53.00 (*1.00*) [a] | 51.00 (*1.00*) [a] | 51.00 (*1.00*) [a] | 56.00 (*1.0*) [A] | 53.00 (*2.00*) [A] | 56.00 (*1.00*) [A] |
| Total C (g $kg^{-1}$ DW soil) | 43.80 *(4.40)* [a] | 45.40 (*17.10*) [a] | 46.10 (*4.80*) [a] | 35.90 (*2.0*) [A] | 37.90 (*0.70*) [A] | 33.60 (*0.50*) [A] |
| DOC (mg $kg^{-1}$ DW soil) | 115.40 *(7.48)* [a] | 105.41 *(7.96)* [a] | 113.45 (1.64) [a] | 82.06 *(5.49)* [A] | 84.06 *(1.48)* [A] | 80.56 *(3.41)* [A] |
| Total N (g $kg^{-1}$ DW soil) | 4.07 *(0.44)* [a] | 4.47 (*0.10*) [a] | 4.59 (*0.41*) [a] | 3.48 (*0.41*) [A] | 3.59(*0.13*) [A] | 3.24 (*0.50*) [A] |
| DON (mg $kg^{-1}$ DW soil) | 55.72 *(14.11)* [a] | 44.71 *(5.11)* [a] | 48.26 *(9.39)* [a] | 45.88 *(5.311)* [A] | 47.38 *(6.71)* [A] | 31.79 *(1.36)* [A] |
| $NH_4^+$-N (mg $kg^{-1}$ DW soil) | 18.17 *(8.58)* [a] | 9.47 (*0.76*) [a] | 12.59 (*3.23*) [a] | 11.82 (*2.08*) [A] | 13.12 (*1.78*) [A] | 11.29 (*1.24*) [A] |
| $NO_2/NO_3^-$ -N (mg $kg^{-1}$ DW soil) | 20.20 *(2.88)* [a] | 16.11 (*3.53*) [a] | 24.59 (*9.55*) [a] | 9.67 (*2.57*) [A] | 6.15(*0.46*) [A] | 6.86 (*1.12*) [A] |
| Total P (P mg $kg^{-1}$ DW soil) | 437.50 (*39.00*) [a] | 739.50 (*64.50*) [b] | 842.80 (*20.1*) [b] | 563.20 (16.9) [A] | 608.9 (*13.5*) [A] | 639.8 (*29.30*) [A] |
| Pi (P mg $kg^{-1}$ DW soil) | 3.13 (*0.24*) [a] | 8.88 (*1.06*) [ab] | 20.83(*2.11*) [b] | 3.08 (*0.14*) [A] | 2.67 (*0.14*) [A] | 4.32 (*0.34*) [B] |

**Table 2**

| | Pi fertilisation (kg $ha^{-1}$ $yr^{-1}$) | Total genera (*S*) | Richness (*d*) | Shannon diversity (*H' log*$^{e}$) |
|---|---|---|---|---|
| Grazed site | P0 | 201 *(26)*$^{a}$ | 44.12 *(4.75)*$^{a}$ | 5.14 *(0.13)*$^{a}$ |
| | P15 | 190 *(13)*$^{a}$ | 42.06 *(2.67)*$^{a}$ | 5.09 *(0.06)*$^{a}$ |
| | P30 | 166 *(15)*$^{a}$ | 37.81 *(2.90)*$^{a}$ | 4.94 *(0.09)*$^{a}$ |
| Cut and removed site | P0 | 154 *(12)*$^{A}$ | 35.68 *(2.41)*$^{A}$ | 4.87 *(0.08)*$^{A}$ |
| | P15 | 150 *(12)*$^{A}$ | 34.70 *(2.31)*$^{A}$ | 4.84 *(0.09)*$^{A}$ |
| | P30 | 223 *(65)*$^{A}$ | 48.78 *(12.30)*$^{A}$ | 5.15 *(0.25)*$^{A}$ |

**Table 3**

**a) Grazed site**

| Genus/Genus-type group | Phylum | Relative abundance (%) | | | Mean relative abundance |
|---|---|---|---|---|---|
| | | G- P0 | G-P15 | G-P30 | (%) |
| ***Gp6*** | **Acidobacteria** | **7.84 (1.54)$^{a}$** | **14.91 (1.47)$^{b}$** | **20.89 (0.37)$^{b}$** | **14.55** |
| *Spartobacteria_genera_incertae_sedis* | Verrucomicrobia | 8.45 (4.30)$^{a}$ | 14.05 (1.46)$^{a}$ | 16.90 (2.19)$^{a}$ | 13.14 |
| ***Gp16*** | **Acidobacteria** | **7.10 (1.18)$^{a}$** | **12.13 (0.49)$^{ab}$** | **15.74 (0.60)$^{b}$** | **11.66** |
| *Prevotella* | Bacteroidetes | 0.06 (0.04)$^{a}$ | 3.69 (3.64)$^{a}$ | 12.07 (9.68)$^{a}$ | 5.28 |
| *Singulisphaera* | Planctomycetes | 3.18 (1.66)$^{a}$ | 5.03 (0.83)$^{a}$ | 3.54 (1.44)$^{a}$ | 3.92 |
| *unclassified_Rhizobiales* | Proteobacteria | 3.88 (1.91)$^{a}$ | 4.05 (0.82)$^{a}$ | 2.45 (0.77)$^{a}$ | 3.46 |
| *unclassified_Bacillaceae_1* | Firmicutes | 3.39 (1.71)$^{a}$ | 3.47 (0.59)$^{a}$ | 2.55 (0.74)$^{a}$ | 3.14 |
| ***unclassified_Actinobacteria*** | **Actinobacteria** | **3.11 (1.53)$^{a}$** | **3.50 (0.78)$^{ab}$** | **2.37 (0.69)$^{b}$** | **3.00** |
| *unclassified_Planctomycetaceae* | Planctomycetes | 2.06 (1.12)$^{a}$ | 2.95 (0.52)$^{a}$ | 2.32 (1.01)$^{a}$ | 2.44 |
| *unclassified_Bacillales* | Firmicutes | 2.29 (1.14)$^{a}$ | 2.69 (0.29)$^{a}$ | 1.56 (0.48)$^{a}$ | 2.18 |
| ***Bradyrhizobium*** | **Proteobacteria** | **3.63 (0.10)$^{a}$** | **0.67 (0.16)$^{b}$** | **0.30 (0.08)$^{b}$** | **2.09** |
| *unclassified_Veillonellaceae* | Actinobacteria | 0.09 (0.05)$^{a}$ | 1.86 (1.80)$^{a}$ | 3.33 (2.36)$^{a}$ | 1.76 |
| *unclassified_Prevotellaceae* | Firmicutes | 0.04 (0.03)$^{a}$ | 2.45 (2.49)$^{a}$ | 2.48 (2.13)$^{a}$ | 1.66 |
| ***unclassified_Acidimicrobiales*** | **Bacteroidetes** | **2.34 (0.07)$^{a}$** | **1.66 (0.34)$^{ab}$** | **0.88 (0.25)$^{b}$** | **1.63** |
| *Gp5* | Acidobacteria | 3.20 (3.09)$^{a}$ | 0.08 (0.02)$^{a}$ | 0.03 (0.01)$^{a}$ | 1.10 |
| *Sporosarcina* | Firmicutes | 1.11 (0.59)$^{a}$ | 1.20 (0.17)$^{a}$ | 0.97 (0.33)$^{a}$ | 1.09 |
| *Acholeplasma* | Tenericutes | 2.94 (1.27)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.98 |
| *Gp7* | Acidobacteria | 1.06 (0.53)$^{a}$ | 1.03 (0.20)$^{a}$ | 0.63 (0.21)$^{a}$ | 0.91 |
| *unclassified_Lachnospiraceae* | Firmicutes | 0.08 (0.02)$^{a}$ | 0.74 (0.67)$^{a}$ | 1.90 (1.41)$^{a}$ | 0.91 |
| *unclassified_Opitutaceae* | Verrucomicrobia | 2.72 (2.71)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.91 |
| *3_genus_incertae_sedis* | Verrucomicrobia | 0.99 (0.52)$^{a}$ | 1.04 (0.17)$^{a}$ | 0.66 (0.16)$^{a}$ | 0.90 |
| *Gp3* | Acidobacteria | 1.29 (0.65)$^{a}$ | 0.95 (0.21)$^{a}$ | 0.25 (0.08)$^{a}$ | 0.83 |
| *Gp15* | Acidobacteria | 2.43 (0.21)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.00 (0.00)$^{a}$ | 0.81 |
| *Nitrospira* | Nitrospira | 2.20 (2.06)$^{a}$ | 0.13 (0.04)$^{a}$ | 0.06 (0.02)$^{a}$ | 0.80 |

**b) Cut and removed site**

| Genus/Genus-type group | Phylum | Relative abundance (%) | | | Mean relative abundance (%) |
|---|---|---|---|---|---|
| | | G- P0 | G-P15 | G-P30 | |
| *Gp6* | Acidobacteria | 19.22 (9.69)[a] | 30.73 (4.73)[a] | 11.73 (7.77)[a] | 19.80 |
| *Gp16* | Acidobacteria | 11.62 (5.71)[a] | 19.15 (3.08)[a] | 8.19 (4.60)[a] | 12.51 |
| *Spartobacteria_genera_incertae_sedis* | Verrucomicrobia | 7.51 (3.78)[a] | 14.51 (1.22)[a] | 9.20 (4.61)[a] | 10.02 |
| *unclassified_Actinobacteria* | Actinobacteria | 2.87 (1.54)[a] | 3.74 (0.75)[a] | 3.13 (1.73)[a] | 3.13 |
| *unclassified_Rhizobiales* | Proteobacteria | 2.24 (1.17)[a] | 3.45 (0.49)[a] | 3.72 (2.22)[a] | 3.02 |
| *Gp5* | Acidobacteria | 4.92 (4.85)[a] | 0.05 (0.03)[a] | 4.16 (4.03)[a] | 2.93 |
| *Singulisphaera* | Planctomycetes | 2.08 (1.05)[a] | 4.10 (0.55)[a] | 2.68 (1.34)[a] | 2.85 |
| *unclassified_Planctomycetaceae* | Planctomycetes | 2.42 (1.30)[a] | 4.04 (0.43)[a] | 2.24 (1.12)[a] | 2.79 |
| *unclassified_Bacillaceae_1* | Firmicutes | 1.79 (1.01)[a] | 2.65 (0.45)[a] | 2.32 (1.40)[a] | 2.17 |
| *unclassified_Bacillales* | Firmicutes | 1.51 (0.92)[a] | 2.87 (0.60)[a] | 2.21 (1.41)[a] | 2.12 |
| *Acholeplasma* | Tenericutes | 2.99 (1.29)[a] | 0.00 (0.00)[a] | 3.34 (3.21)[a] | 2.03 |
| *unclassified_Opitutaceae* | Verrucomicrobia | 2.69 (1.64)[a] | 0.01 (0.00)[a] | 2.88 (2.29)[a] | 1.79 |
| *Gp15* | Acidobacteria | 2.56 (2.01)[a] | 0.02 (0.01)[a] | 2.78 (1.85)[a] | 1.72 |
| *unclassified_Acidimicrobiales* | Actinobacteria | 1.46 (0.36)[a] | 1.31 (0.24)[a] | 1.98 (0.38)[a] | 1.53 |
| *Nitrospira* | Nitrospira | 1.63 (1.50)[a] | 0.13 (0.03)[a] | 1.90 (1.68)[a] | 1.18 |
| *3_genus_incertae_sedis* | Verrucomicrobia | 0.92 (0.47)[a] | 1.54 (1.54)[a] | 1.05 (0.54)[a] | 1.12 |
| *Phycisphaera* | Planctomycetes | 1.50 (1.05)[a] | 0.00 (0.00)[a] | 1.66 (0.31)[a] | 1.01 |
| *Gp17* | Acidobacteria | 1.06 (0.55)[a] | 1.57 (0.37)[a] | 0.52 (0.34)[a] | 1.01 |
| *Holophaga* | Acidobacteria | 1.34 (1.24)[a] | 0.00 (0.00)[a] | 1.70 (1.24)[a] | 0.98 |
| *Gp7* | Acidobacteria | 0.73 (0.39)[a] | 1.65 (0.42)[a] | 0.53 (0.36)[a] | 0.93 |
| *Gp4* | Acidobacteria | 0.90 (0.46)[a] | 1.07 (0.06)[a] | 0.58 (0.27)[a] | 0.82 |
| *Sporosarcina* | Firmicutes | 0.56 (0.31)[a] | 0.99 (0.22)[a] | 0.75 (0.48)[a] | 0.74 |
| *unclassified_Actinomycetales* | Actinobacteria | 0.55 (0.28)[a] | 0.94 (0.17)[a] | 0.78 (0.40)[a] | 0.73 |
| *Zavarzinella* | Planctomycetes | 0.86 (0.53)[a] | 0.40 (0.04)[a] | 0.83 (0.55)[a] | 0.67 |

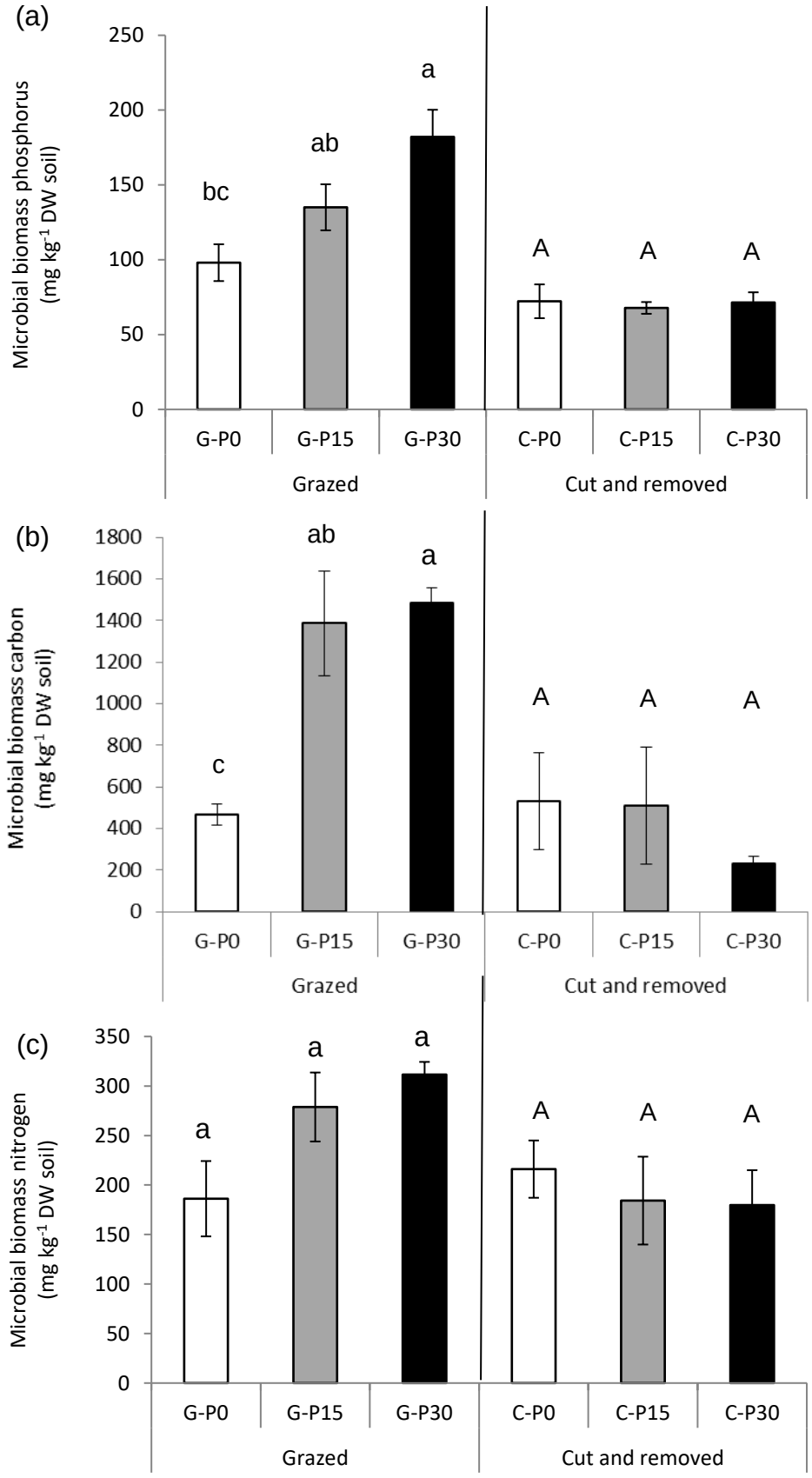

(a)
Microbial biomass phosphorus (mg kg-1 DW soil)
250
200
150
100
50
0
bc
ab
a
A
A
A
G-P0
G-P15
G-P30
C-P0
C-P15
C-P30
Grazed
Cut and removed
(b)
Microbial biomass carbon (mg kg-1 DW soil)
1800
1600
1400
1200
1000
800
600
400
200
0
c
ab
a
A
A
A
G-P0
G-P15
G-P30
C-P0
C-P15
C-P30
Grazed
Cut and removed
(c)
Microbial biomass nitrogen (mg kg-1 DW soil)
350
300
250
200
150
100
50
0
a
a
a
A
A
A
G-P0
G-P15
G-P30
C-P0
C-P15
C-P30
Grazed
Cut and removed

**Fig 1**

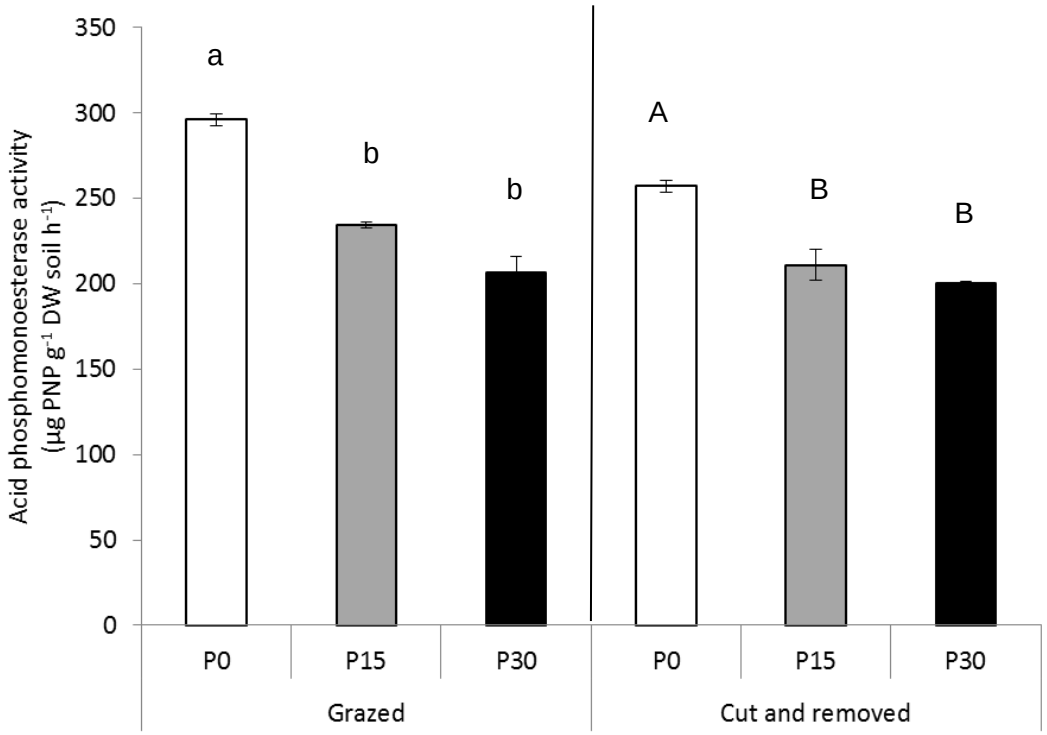

Acid phosphomonoesterase activity
(µg PNP g-1 DW soil h-1)
350
300
250
200
150
100
50
0
a
b
b
A
B
B
P0
P15
P30
P0
P15
P30
Grazed
Cut and removed

**Fig 2**

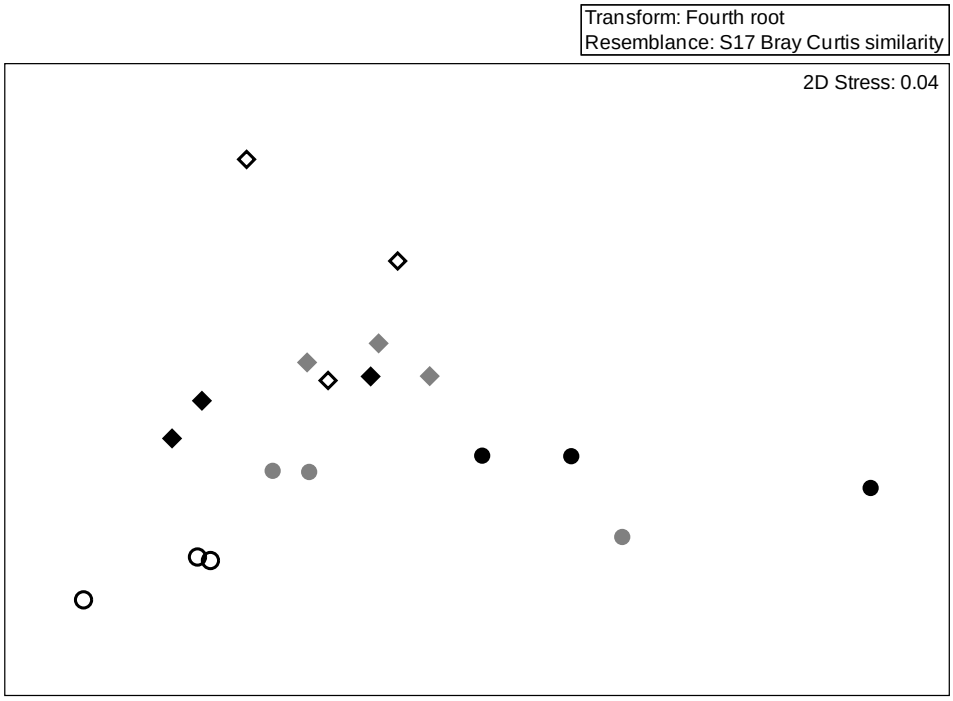

Transform: Fourth root
Resemblance: S17 Bray Curtis similarity
2D Stress: 0.04

**Fig 3**

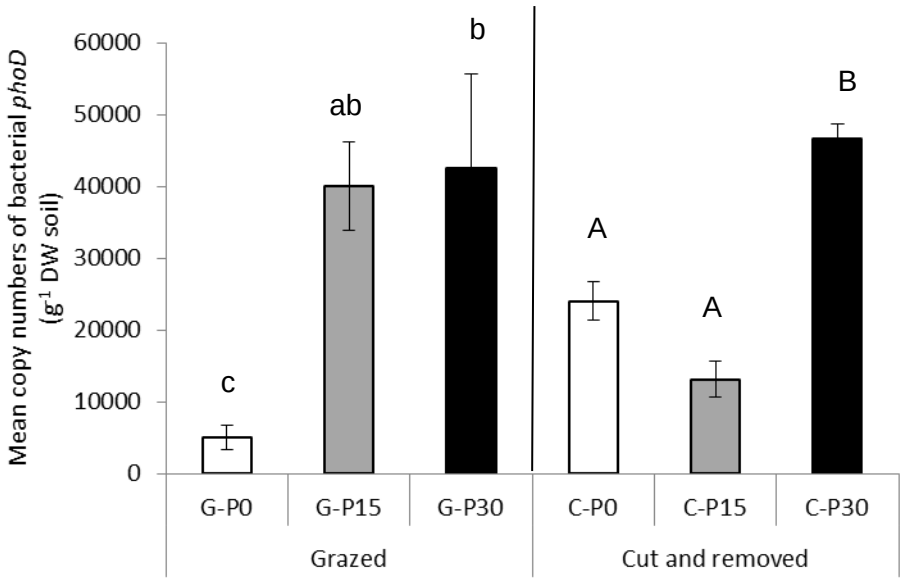

Mean copy numbers of bacterial phoD (g-1 DW soil)
60000
50000
40000
30000
20000
10000
0
c
ab
b
A
A
B
G-P0
G-P15
G-P30
C-P0
C-P15
C-P30
Grazed
Cut and removed

**Fig 4**

**Table titles and figure legends**

**Table 1** Soil physicochemical parameters for soil at the grazed (G) and cut and removed (C) sites receiving long-term additions of 0 (P0), 15 (P15) or 30 (P30) kg $ha^{-1}$ $yr^{-1}$ of inorganic phosphorus**.** Significant differences across rows are denoted by different letters ($P<0.05$). Lower case letters indicate differences across the grazed site. Capital letters indicate differences across the cut and removed site ($p<0.05$). Standard errors of the mean are shown in parentheses.

**Table 2** Diversity measurements of soil bacterial OTUs classified at genus/genus-type level receiving long-term additions of 0 (P0), 15 (P15) or 30 (P30) kg $ha^{-1}$ $yr^{-1}$ of inorganic phosphorus at the grazed and cut and removed grassland sites. DNA was extracted in triplicate from three plots for each application rate at each site. Data was generated using the DIVERSE function in PRIMER-E. Significant differences are denoted by different letters within columns ($p<0.05$). Lower case letters indicate differences across the grazed site, while capital letters indicate differences across the cut and removed site.

**Table 3** Top 24 bacterial genera/genus-type based on mean relative abundances in a) plots at the grazed (G) site exposed to Pi fertilisation rates (G-0, G-15 and G-30 kg $ha^{-1}$ $yr^{-1}$) and b) plots at the cut and removed (C) site exposed to inorganic phosphorus fertilisation rates (C-0, C-15 and C-30 kg $ha^{-1}$ $yr^{-1}$). Differences in letters within rows indicate significant differences in individual genera ($p<0.05$). Bacterial genera/genus-type exhibiting significant differences in relative abundance are highlighted in bold. Standard errors of the mean are shown in parentheses.

**Fig 1** Mean (n=3) microbial biomass (a) phosphorous, (b) carbon and (c) nitrogen measured in plots receiving long-term inorganic phosphorus fertilisation at 0 (P0) (white bars), 15 (P15) (grey bars) or 30 (P30) (black bars) kg $ha^{-1}$ $yr^{-1}$ across the grazed and cut and removed grassland sites. Error bars represent standard errors of the mean. Lower case letters indicate differences across the grazed site. Capital letters indicate differences across the cut and removed site ($p<0.05$).

**Fig 2** Mean (n=3) acid phosphomonoesterase activity measured soil from triplicate plots receiving long-term inorganic phosphorus fertilisation at 0 (P0), 15 (P15) or 30 (P30) kg $ha^{-1}$ $yr^{-1}$ across the grazed and cut and removed grassland sites. Error bars represent standard errors of the mean. Lower case letters indicate differences across the grazed site. Capital letters indicate differences across the cut and removed site ($p<0.05$).

**Fig 3** NMDS plot visually representing bacterial community structures profiled using targeted amplicon sequencing of the bacterial *16S rRNA* gene in soil exposed to long-term differences in inorganic phosphorus fertilisation (n=3) at the grazed (G) site: unfertilised (G-P0) (white circles), 15 (G-P15) (grey circles) or 30 (G-P30) (black circles) kg $ha^{-1}$ $yr^{-1}$ and at the cut and removed (C) site: unfertilised (C-P0) (white diamonds), 15 (C-P15) (grey diamonds) or 30 (C-P30) (black diamonds) kg $ha^{-1}$ $yr^{-1}$.

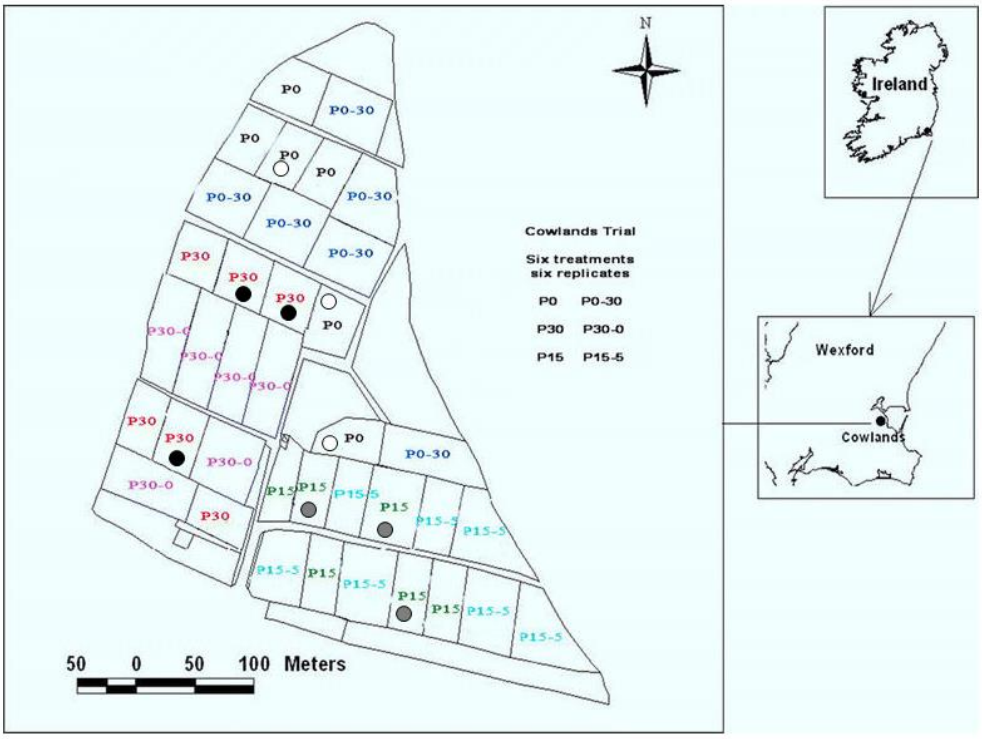
**Fig 4** Mean (n=3) copy numbers of a bacterial alkaline phosphatase gene *phoD* from triplicate plots receiving long-term inorganic phosphorus fertilisation at 0 (P0), 15 (P15) or 30 (P30) kg $ha^{-1}$ $yr^{-1}$ across the (a) grazed and (b) cut and removed grassland sites. Error bars represent standard errors of the mean. Lower case letters indicate differences across the grazed site. Capital letters indicate differences across the cut and removed site ($p<0.05$).

**Supplementary material**

**SI Table 1**

| | LN $NH_4^+$ | $NO_3^-$ | Total C | Ki | Total N | Total P | Pi | LN pH | †MC | ††MB C:P | ††MB N:P | ††MB C:N | Soil C:N | Soil C:P | Soil N:P | DOC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LN $NH_4^+$ | | | | | | | | | | | | | | | | |
| $NO_3^-$ | 0.45 | | | | | | | | | | | | | | | |
| Total C | 0.16 | 0.38 | | | | | | | | | | | | | | |
| Ki | 0.48 | 0.24 | -0.11 | | | | | | | | | | | | | |
| Total N | 0.11 | 0.42 | **0.98** | -0.16 | | | | | | | | | | | | |
| Total P | -0.11 | 0.28 | 0.37 | -0.24 | 0.51 | | | | | | | | | | | |
| Pi | -0.06 | 0.51 | 0.48 | -0.22 | 0.58 | **0.80** | | | | | | | | | | |
| LN pH | -0.15 | -0.51 | -0.34 | -0.26 | -0.31 | 0.21 | -0.10 | | | | | | | | | |
| †MC | 0.16 | -0.26 | -0.27 | 0.25 | -0.31 | 0.06 | -0.10 | 0.45 | | | | | | | | |
| ††MB C:P | -0.13 | 0.08 | 0.19 | 0.33 | 0.24 | 0.25 | 0.15 | -0.29 | 0.07 | | | | | | | |
| ††MB N:P | 0.22 | -0.38 | -0.24 | 0.50 | -0.24 | -0.14 | -0.36 | 0.23 | 0.66 | 0.34 | | | | | | |
| ††MB C:N | -0.26 | 0.44 | 0.45 | -0.11 | 0.55 | 0.53 | 0.61 | -0.32 | 0.04 | 0.49 | -0.11 | | | | | |
| Soil C:N | 0.17 | -0.20 | 0.01 | 0.19 | -0.21 | -0.62 | -0.49 | -0.05 | 0.18 | -0.27 | -0.03 | -0.44 | | | | |
| Soil C:P | 0.24 | 0.12 | 0.34 | 0.18 | 0.19 | -0.72 | -0.37 | -0.53 | -0.26 | -0.17 | -0.12 | -0.17 | 0.63 | | | |
| Soil N:P | 0.23 | 0.15 | 0.38 | 0.16 | 0.25 | -0.69 | -0.34 | -0.57 | -0.31 | -0.14 | -0.12 | -0.12 | 0.53 | **0.99** | | |
| DOC | 0.16 | **0.72** | **0.74** | -0.04 | **0.72** | 0.26 | 0.48 | -0.57 | -0.37 | 0.03 | -0.54 | 0.53 | 0.01 | 0.36 | 0.39 | |
| DON | **0.71** | 0.67 | 0.42 | 0.29 | 0.38 | -0.04 | 0.16 | -0.32 | 0.07 | 0.06 | 0.04 | 0.12 | 0.11 | 0.42 | 0.44 | 0.40 |

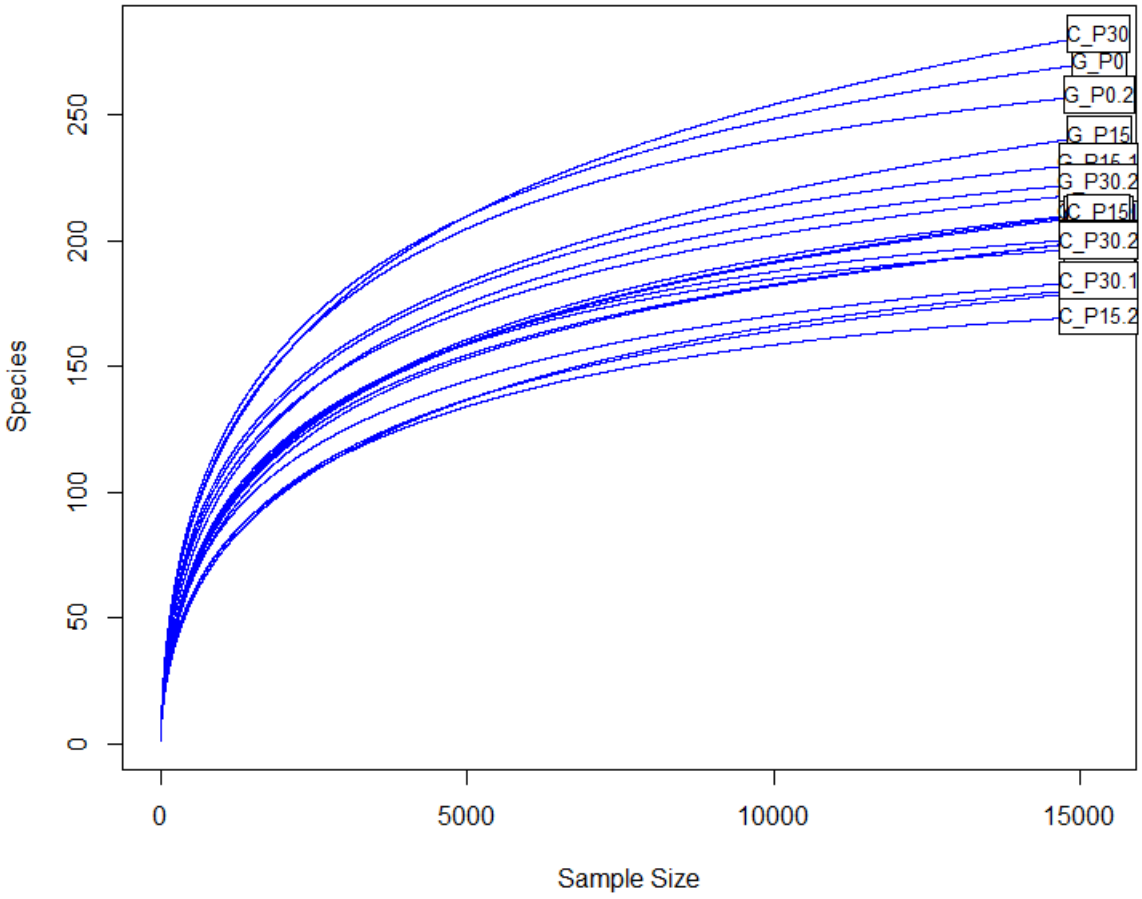
## Figures




**SI Fig 1**

| | | | |
|---|---|---|---|
| P30 | P15 | P45 | P15 |
| P45 ● | P0 ◍ | P0 | P0 ◍ |
| P0 | P30 | P15 ○ | P45 ○ |
| P15 ○ | P45 ● | P0 ◍ | P30 ● |

**SI Fig 2**


C_P30
G_P0
G_P0.2
G_P15
G_P30.2
C_P30.2
C_P30.1
C_P15.2
Species
0
50
100
150
200
250
0
5000
10000
15000
Sample Size

**SI Fig 3**

**Supplementary material table title and figure legends**

**SI Table 1** $R^2$ correlations between measured soil parameter generated in PRIMER-E. LN = log transformed variables. †MC = moisture content, ††microbial biomass, DOC = dissolved organic C; DON = dissolved organic N; Pi = available inorganic phosphorus. Values in bold indicate parameters which covary ($R^2$>0.7).

**SI Fig 1** Adapted map of the grazed (G) site (Griffiths et al., 2012) detailing plot location and associated long-term inorganic phosphorus (Pi) fertilisation rates since establishment in 1968 and post 1999. Plots sampled are indicated by shapes; white circles indicate the unfertilised (G-P0) plots, grey circles indicate G-P15 plots and black circles indicate the G-P30 plots. Fertilisation rates represent 0, 15 or 30 kg $ha^{-1}$ $yr^{-1}$ of Pi.

**SI Fig 2** Diagram of the experiment at the cut and removed (C) site detailing plot location and long-term inorganic phosphorus (Pi) fertilisation rates received since t establishment in 1995. Fertilisation rates represent either 0 (C-P0) (white circles), 15 (C-P15) (grey circles), 30 (C-P30) (black circles) or 45 (P45) kg $ha^{-1}$ $yr^{-1}$ of Pi. All plots from C-P0, C-P15 and C-P30 were sampled, but only those indicated by circles were used to equal that of the grazed site. P45 plots were not sampled.

**SI Fig 3** Rarefaction curves showing the relationship between sampling intensity and the number of recovered sequences using an Illumina MiSeq sequencing platform for soil of triplicate plots receiving long-term Pi fertilisation at 0 (P0), 15 (P15) or 30 (P30) kg $ha^{-1}$ $yr^{-1}$ across the grazed (G) and cut and removed (C) grassland sites.